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Functional studies of Arabidopsis annexins AnnAt1 and AnnAt2 primary root growth

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Functional studies of Arabidopsis annexins AnnAt1 and AnnAt2 primary root growth

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Abstract

Functional studies of Arabidopsis annexins AnnAt1 and AnnAt2 primary root growth

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Annexins are a multigene family of calcium-dependent membrane-binding proteins that play important roles in plant cell signaling. Annexins are multifunctional proteins, and their function in plants is not comprehensively understood. Arabidopsis (*Arabidopsis thaliana*) annexins ANN1 and ANN2 are 64% identical in their primary structure, and both are highly expressed in seedlings. Here, we showed that null mutants of *AnnAt1* or *AnnAt2* grown as seedlings in the absence of sugar show decreased primary root growth and altered columella cells in root caps; but these mutant defects are rescued by sucrose, glucose, or fructose. In seedlings grown without sugar, significant up-regulation of genes encoding proteins needed for photosynthesis and increased chlorophyll accumulation was found in the cotyledons of the null mutants compared to in wild-type, which indicates potential sugar starvation in the roots of the mutant seedlings. Unexpectedly, the overall sugar content of primary roots was significantly higher in roots of the mutants compared to wild-type when the seedlings were grown without sugar for a week. To examine the diffusion of sugar along the entire root to the root tip, we examined the unloading pattern of carboxyfluorescein dye and found that post-phloem sugar transport was impaired in the mutant root tips compared to that in wild type. Also, in the root tips of the mutant seedlings grown without sucrose we detected increased levels of ROS and callose, the latter of which might restrict plasmodesmal sugar transport to root tips. These results indicate that ANN1 and ANN2 play an

important role in post-phloem sugar transport to the root tip, which in turn might indirectly influence photosynthesis in cotyledons, as suggested by observed changes in chlorophyll content and expression levels of photosynthetic genes. Using transmission electronic microscopy, we observed structural alterations in the plasmodesmata of post-phloem root cells of the null mutants, and using RNA-seq and gene ontology analyses we found that knocking out *ANN1* and *ANN2* induced global transcriptomic changes in Arabidopsis roots. This study expands our understanding of the function of annexins in plants and sheds light on the role of plasmodesmata in post-phloem transport and intercellular signaling.

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Chapter 1

Introduction

My dissertation research has discovered a novel functional connection between the expression of two annexins, AnnAt1 and AnnAt2, and the plasmodesmatal transport of sugar in the control of root growth in Arabidopsis. My results indicate potential roles of these annexins in regulating primary root growth and post-phloem transport of sugar. As background information for my dissertation research, here I will provide a brief introduction on how sugar signaling and annexins could affect plant growth generally, and, more specifically, primary root growth in Arabidopsis seedlings.

Critical roles of sugar in plant growth and development

Plant growth is a highly energy-demanding process. Growth is favored by plants under optimum conditions and restricted in unfavorable conditions, like attack by pathogens and exposure to abiotic stresses, leading to energy and nutrient stresses. Both growth promotion and growth inhibition require plants to integrate environmental signals and energy levels to adapt to the ever-changing environment. Sugars are key indicators of plants' energy levels. In addition to serving as energy suppliers and cell structure constituents, sugars are also hormone-like signal molecules which cross-talk with hormone signaling networks, nutrient signals and light signals to regulate gene expression and physiology during plant growth and development (Moore et al., 2003; Ljung et al., 2015). For example, independent of its osmotic effects, 100 mM exogenous sucrose is enough to inhibit hypocotyl growth in darkness, whereas root growth is unaffected at the same concentration (Kircher and Schopfer, 2012).

Microarray studies reveal sugars affect the expression of a broad spectrum of genes that

control processes ranging from carbohydrate metabolism and signal transduction to the transport of metabolites (Price et al., 2004; Thum et al., 2004). Some ethylene biosynthetic genes and signaling genes, such as *CTR1*, *EIN3* and *EIL1,* are downregulated by glucose (Price et al., 2004). Sucrose and auxin interact together to modulate plant responses to Fe deficiency (Lin et al., 2016). Microarray data from Arabidopsis seedlings treated with glucose and the protein synthesis inhibitor cycloheximide (CHX) show that CHX barely affects which genes are repressed by glucose, whereas it greatly decreases the number of genes upregulated by glucose (Price et al., 2004). CHX functions to block *de novo* protein synthesis but enhances mRNA stability. Taking these results together, the regulatory mechanisms induced by glucose may include both posttranscriptional and transcriptional processes.

Sugar molecules include glucose, fructose, trehalose, sucrose and their derivatives. In plants, photosynthesis produces triose phosphates that are stored as starch in chloroplasts or converted into sucrose, which is then either stored in vacuoles or split into glucose and fructose by invertases, or into UDP-glucose and fructose by sucrose synthases (Rolland et al., 2006). Emerging evidence shows that different sugar signals appear to have different functions through distinct signaling pathways. In the next few paragraphs I will briefly summarize what is known about glucose, fructose and sucrose signaling.

The signaling pathways induced by glucose derived from the degradation of starch or sucrose are among the best studied. Based on studies thus far, at least three glucose signaling pathways exist in plants (Xiao et al., 2000). Two of these pathways begin with the sensor activity of hexokinase 1 (HXK), the first sugar receptor discovered in Arabidopsis (Jang et al., 1997). In the first pathway, glucose is sensed and phosphorylated by HXK to regulate the expression of photosynthesis-related genes. The second pathway is glycolysis-dependent. Glucose is catalyzed into glycolytic intermediates. One or several intermediates activate downstream signaling pathways regulating gene expression. Both of the first two pathways depend on the function of

HXK. In the third pathway, glucose is sensed by other sensors that have not been discovered yet. Two distinct effects of HXK-mediated glucose signaling have been revealed (Sheen, 2014). One is to function as a feedback loop for sugar production by repressing photosynthesis and leaf development. The other one is to promote early organ growth and expansion of newly divided cells.

Hexokinase is a dual functioning protein with both glucose catalytic activity and glucose-sensing activity. Glucose is first phosphorylated into glucose-6-phosphate by HXK and then either broken down into glycolytic products or used as a signal to change the expression of photosynthesis-related genes. Application of 2-deoxy-D-glucose (2-dG) can block its glucose catalytic activity and uncouple glucose signaling from metabolism. Treatment with 2-dG still induces glucose phosphorylation and represses the expression of photosynthesis genes. The sensor activity of HXK independent of its catalytic function has also been confirmed by the discovery of two catalytically inactive mutants (*At*HXK1^{G104D} and *At*HXK1^{S177A}) that still maintain the sugar-sensing function to regulate gene expression (Moore et al., 2003).

Fructose comes from the hydrolysis of sucrose. High fructose treatment of Arabidopsis seedlings results in early developmental arrest with inhibited hypocotyl and root growth and repressed cotyledon expansion and chlorophyll accumulation (Cho and Yoo, 2011). Also, the non-metabolizable fructose analog psicose inhibits root growth in lettuce. A fructose sensor, a putative fructose-1, 6-bisphosphatase (FINS1/FBP), discovered in Arabidopsis, reveals a regulatory role for fructose and shows this pathway is distinct from those initiated by glucose (Cho and Yoo, 2011; Sheen, 2014). Treatment of plants with a catalytically inactive form FBPS^{126A,S127A} still suppresses photosynthetic gene expression in the same manner as the wild-type FBP. This indicates a regulatory role of the putative FBP in fructose signaling that is independent of its catalytic activity in sugar metabolism, which is similar to how HXK1 functions in glucose signaling. ANAC089, an OsNAC8 subgroup member of NAC transcription factors, has been identified in the fructose-signaling pathway, but not in the glucose-signaling pathway (Li et al., 2011). The constitutive

expression of *ANAC089* gene results in fructose insensitivity. How the fructose receptor FINS1/FBP potentially works with the NAC transcription factor remains unclear.

Sucrose is the main transport sugar in plants. Its regulatory roles as a signal molecule have been neglected for many years, mainly because sucrose can be catalyzed by invertase into glucose and fructose. It is hard to tell whether sucrose is the direct signal to regulate plant growth and gene expression, or it works through the regulatory functions of glucose and fructose. Sucrose sensors have not been discovered yet, but it seems clear that high concentrations of sucrose induce an increase in cytoplasmic calcium levels that act as a second messenger. Sucrose and glucose signaling converge at the SnRK and rapamycin (TOR) kinase signaling pathways (Lastdrager et al., 2014). SnRK and TOR are two key regulatory networks in plants to monitor sugar status. SnRK is activated in sugar starvation conditions, while TOR promotes plant growth in high sugar conditions. The mechanism by which these two pathways coordinate together to regulate plant growth remains unclear. In summary, sugars are important plant growth regulators, and small changes in their level can significantly alter plant physiology and adaptations to environment.

Multifunctional annexins in plants

Annexins are multifunctional proteins that are involved in many growth and development processes, including stress responses (Clark et al., 2012). They are differentially expressed in all tissues and can be distributed throughout the plant from their site of production through phloem translocation. In chapter 2, I will discuss the many diverse functions attributed to plant annexins, but here I will introduce the role of certain plant annexins in Ca^{2+} transport. Unlike other Ca^{2+}-permeable channels, certain plant annexins are suggested to form "unconventional" Ca^{2+} channels (Davies, 2014). Ca^{2+} permeable channel proteins typically have transmembrane domains and annexins do not, so it is still uncertain whether certain annexins act directly as Ca^{2+} channels or simply facilitate Ca^{2+} influx by regulating Ca^{2+} channels. While all other Ca^{2+}-permeable channel

proteins are targeted to specific membranes through vesicle delivery, annexins seem able to associate with membranes in multiple subcellular locales including lipid rafts. Therefore, it is hypothesized that annexins could be recruited directly to membranes, independent of vesicle delivery, in response to multiple stimuli.

In addition, strong evidence indicates that plant annexins are able to bind membranes dependent or independent of calcium and facilitate calcium transport in response to various stresses. In this way plant annexins can serve as calcium signal amplifiers (Davies, 2014). For example, malondialdehyde, which is induced by diverse stresses via the peroxidation of polyunsaturated fatty acids, promotes ZmANN33/35 Ca^{2+} transport activity *in vitro* (Laohavisit et al., 2009), and this, in turn, would have implications for increasing the $[Ca^{2+}]_{cyt}$ signal *in vivo* in response to stresses (Laohavisit et al., 2010). In addition, hydroxyl radicals ($OH^{\bullet}$) are the most stable of reactive oxygen species (ROS) generated in plants. They evoke a significantly different $[Ca^{2+}]_{cyt}$ responses in whole roots compared to those in root epidermal protoplasts (Laohavisit et al., 2012; Richards et al., 2014). That means annexins may also be involved in forming specific calcium signatures, but the mechanism requires further study (Davies, 2014).

My dissertation focuses primarily on the role of annexins in mediating the post-phloem transport of sugars to the tips of primary roots of Arabidopsis seedlings. To understand the novelty and significance of this work, here we will describe the anatomical and transport factors involved in moving sugars from aerial tissues to the tip of primary roots.

Long distance transport

Phloem connects source organs and sink organs that are distant from each other. In addition to moving solutes, phloem tissues transport such macromolecules as RNA and proteins (Turgeon and Wolf, 2009; Turnbull and Lopez-Cobollo, 2013). The translocatable transcripts in Arabidopsis

have been identified by grafting two ecotypes (Thieme et al., 2015), while assays of phloem sap have been used to discover large numbers of proteins in different species, as reported by Turnbull and Lopez-Cobollo (2013). The high velocity of its flow (Windt et al., 2006) and its complex composition make phloem a suitable vehicle for long distant communications in plants. Supporting this hypothesis, the small protein FLOWERING LOCUS T (FT) (19.8KD), which serves as a flowering signal, is translated in leaves and then moves through phloem to the apical meristem to promote flowering (Giakountis and Coupland, 2008). The long-distance transport of FT is mediated by its interaction with heavy-metal-associated domain-containing protein, SODIUM POTASSIUM ROOT DEFECTIVE 1 (NaKR1) in phloem of leaves (Zhu et al., 2016). Long-distance phloem flow also plays a critical role in pathogen response. Mobile protein signals are translocated to distant plant organs where they induce systemic acquired resistance (Fu and Dong, 2013).

Many of the translocated macromolecules have no functional significance and enter the phloem flow by default (Turnbull and Lopez-Cobollo, 2013; Paultre et al., 2016). One of the possible reasons why diverse non-functional macromolecules enter the translocation stream is to maintain the turgor gradient in the pressure flow mechanism of phloem movement from source to sink (Paultre et al., 2016). Many proteins are loaded into sieve elements and unloaded from protophloem, however some are trapped within the pericycle without going into the endodermis (Paultre et al., 2016). Proteins not drawn into the translocation flow have several characteristics (Paultre et al., 2016): 1) proteins translated on ER-bound ribosomes are not able to enter the translocation stream; 2) the translocation of proteins with molecular mass above 70 KD is greatly reduced.

Sugar transport

Photoassimilate moves from mesophyll cells to phloem sieve element and companion cells, by a process referred to as phloem loading. There are three different loading strategies: 1) Passive

symplastic transport through plasmodesmata; 2) active symplastic transport through polymer trapping; 3) apoplastic transport by sugar transporters. According to the preference of sugar loading strategies, plants are categorized into 3 types: type 1, type 1-2a, type 2 (Davidson et al., 2011; Liesche, 2017). Type 1 prefers a combination of both active and passive symplastic transport, while type 2 favors apoplastic loading. Arabidopsis falls into type 1-2a using all three sugar loading methods. In Arabidopsis, sugar diffuses through plasmodesmata from bundle sheets into phloem parenchyma cells where its efflux into the apoplast is mediated by sugar transporters such as SWEET and SUC. Then sugar is retrieved into phloem sieve element and companion cells by sugar transporters such as SUS and SUT (Haritatos et al., 2000; Chen et al., 2012; Julius et al., 2017).

Depending on the type of sink in roots, there are two ways for sugar to exit phloem. In growing root tips symplastic unloading through plasmodesmata dominates. In carbon storage roots, apoplastic unloading dominates, and the sugar is unloaded by sugar transporters such as SWEET and SUC and then converted by cell wall invertase into hexoses in the apoplast. Eventually, hexoses are taken up by transporters into root cells (Hennion et al., 2019).In post-phloem cells, symplastic and apoplastic modes of unloading both happen depending on the diverse ways sugar is used and metabolized.

Under experimental *in vitro* conditions, such as those used in the research described here, sucrose is routinely added to agar plates and passively diffuses into root tip cells until it reaches the casparian strip. There the added sucrose is catalyzed into fructose and glucose and then taken up into cells by transporters.

Anatomy of post-phloem region

The root as a typical sink organ is of great value for studies in phloem transport. Phloem sieve elements (SE) are long, tube shaped cells that are devoid of most cellular organelles and are specialized for fast movement of both small molecules and macromolecules. SE are connected to companion cells through a large number of plasmodesmata. Each of the two phloem files in roots consists of one metaphloem and one protophloem. Metaphloem functions above the root meristem and connects ultimately to sink organs, while functional protophloem starts near the meristem, beyond which further transport to the root tip is defined as post-phloem (Figure 1). Both metaphloem and protophloem are supported by two companion cells attached to them. Two phloem pole pericycle cells sit outside of metaphloem and inside of endodermal cells (Ross-Elliott et al., 2017; Truernit, 2017). Small molecules and macromolecules are loaded into metaphloem and then transferred to protophloem from which they get unloaded into phloem pole pericycle cells (Figure 1). Small molecules like sugar and free GFP protein diffuse freely through phloem pole pericycle cells into all other cells in the post-phloem region (Fisher and Oparka, 1996). However, many proteins are trapped in phloem pole pericycle cells without going further (Stadler et al., 2005; Paultre et al., 2016; Ross-Elliott et al., 2017). Exceptional proteins like transcription factor SHORT ROOT can be transported into the endodermis, however, the transport mechanism remains to be studied. Whether this is a mechanism used by plants to distinguish functional signals from the non-functional ones and what further passage methods are used to transport functional signals remain unknown.

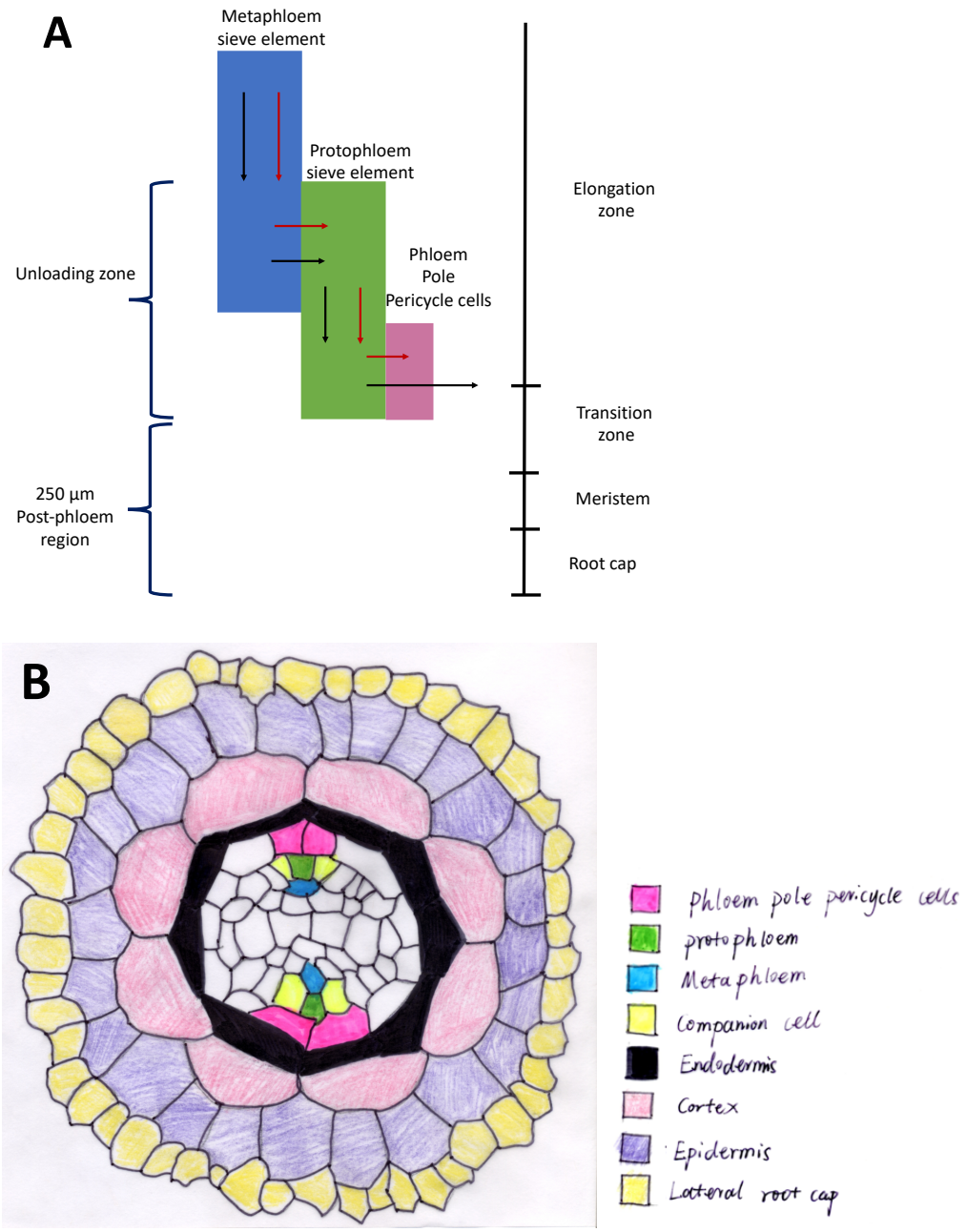

Figure 1.1 Diagrams illustrating root tip cell types and paths of unloading and post-phloem transport of solute in the root. A. Model of unloading and post-phloem transport of sugar and proteins in the root. Both sugar and proteins loaded into metaphloem sieve elements are transferred into protophloem sieve elements. After it, solute is

transported into phloem pole pericycle cells through plasmodesmata. Sugar diffuses freely out of

phloem pole pericycle cells into cells in post-phloem region, while many proteins are trapped in phloem pole pericycle cells unless unknow mechanisms are used. Black arrow: sugar. Red arrow: proteins. B. Cross section of root unloading zone.

Figure 1.1 continued

Chapter 2[1]

AnnAt1 and AnnAt2 function in post-phloem sugar transport in root tips to affect primary root growth

2.1 Introduction

Annexins are multifunctional proteins that have been found in all plants and vertebrate species. Their tertiary structure with calcium-binding domains is evolutionarily conserved in all species (Morgan et al., 2004; Morgan et al., 2006; Konopka-Postupolska et al., 2011; Clark et al., 2012). In plants, annexins have been localized in many parts of the cell, including the plasma membrane, the Golgi-apparatus, secretory vesicles, vacuolar membranes and the nucleus. Certain annexins are also found in the extracellular matrix as secreted proteins.

On the molecular level, certain plant annexins serve as components of calcium channels or regulate calcium channel activity to help initiate and amplify calcium signals in plants (Laohavisit et al., 2010; Laohavisit and Davies, 2011; Davies, 2014). On the cellular level, one of the earliest functions discovered for annexins in plant cells was meditation of vesicle trafficking and secretion (Konopka-Postupolska and Clark, 2017). On the whole-plant level, key discoveries of annexin function have been carried out using mutants of the model plant, Arabidopsis thaliana, where there are eight different annexins that show differential expression patterns (Cantero-Garcia et al., 2006). Several of these mutant studies show that certain Arabidopsis annexins function to provide stress tolerance in response to a variety of stress conditions (Liao et al., 2017; Wang et al., 2015; Dalal et al., 2014; Richards et al., 2014; Konopka-Postupolska et al., 2009). For example, overexpression of AnnAt1 in Arabidopsis results in increased antioxidant activity and confers drought tolerance (Konopka-Postupolska et al., 2009).

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[1]This Chapter was published in Wang et al. (2018) *Plant Physiol.***178:** 390-401. Jing Wang designed and performed the experiments. Jing Wang wrote the manuscript.

Plant annexins are expressed in all stages of plant growth and development. Recently, Thieme et al. (2015) found that some Arabidopsis annexin transcripts can be transported across the whole plant by phloem sap. Annexin proteins have also been identified in the phloem sap of several different plant species (Kehr, 2006), and in Arabidopsis, AnnAt1 was found in phloem exudate of rosette leaves (Guelette et al., 2012). Immunolocalization of a pea annexin protein in young developing sieve elements led to the suggestion that annexins may play a role in phloem cell development (Clark et al., 1992). In situ transcript localization studies found that *AnnAt1* is expressed in phloem and phloem parenchyma in Arabidopsis seedlings (Clark et al., 2001). However, the physiological importance of annexins in phloem remains undefined.

Phloem transport links sources to sinks and is a key passageway for communication between leaves and roots. Sugars generated from photosynthetic leaves (source organs) and transported through the phloem to roots (sink organs) serve both as metabolic substrates to fuel plant growth and development, and as signals that integrate plant responses to environmental stimuli, hormones, and changes in nutrition status (Martin et al., 2002; Moore et al., 2003; Smeekens et al., 2010; Lastdrager et al., 2014; Ljung et al., 2015; Dobrenel et al., 2016; Dodds and Lagudah, 2016; Kuhn, 2016; Baena-Gonzalez and Hanson, 2017, Li and Sheen, 2016). Plants have developed complex sugar-sensing systems to monitor and respond to continuous fluctuations in sugar status caused by the circadian clock and frequent environmental changes (Stitt and Zeeman, 2012).

Multiple recent studies have revealed significant roles of sugar in plant growth (Lastdrager et al., 2014) and development (Wingler, 2017). For example, sugar availability to axillary buds is a key determinant for shoot branching. Removal of pea shoot tips induces a sufficient flow of sugar into axillary buds to repress the expression of the branching inhibitor gene *Branched1* (*BRC1*), which highlights the importance of sugars in mediating apical dominance (Mason et al., 2014). Sugars in photosynthetic leaves function as mobile signals to repress the expression of

microRNA156 (miR156), and thus promote the maturation of juvenile plants (Yu et al., 2013).

Important sugar responses are also found in roots, whose growth depends on sucrose transported from photosynthetic leaves. This dependence underscores the importance of understanding the effects of sugars on root growth and development. Although sugar controls primary and lateral root growth in a dose-dependent manner (Freixes et al., 2002), sugar demand in roots continuously changes due to environmental signals (Lemoine et al., 2013) and developmental stages. Having a sugar-sensing system in roots that can efficiently monitor sugar levels would be important for root and whole plant growth. However, studies of sugar signaling in roots are generally scant.

Root growth is closely associated with sugar concentrations (Thompson et al., 2017). Greater soluble sugar content has been found in fast-growing roots of pruned barley plants, whereas slow-growing barley roots contain less soluble sugar (Farrar and Jones, 1986). Sucrose generated by photosynthesis in cotyledons is required to regulate primary root growth (Kircher and Schopfer, 2012). Sucrose promotes primary root elongation in a dose-dependent manner. The higher the exogenous sucrose concentration, the faster primary roots elongate (Freixes et al., 2002). Furthermore, the finding that neighboring secondary roots in the same root system have different growth rates in their elongation zone as a function of local sugar concentration also favors the conclusion that sugar is a major determinant of root growth (Freixes et al., 2002).

Because local sugar concentrations play critical roles in regulating root growth, it is important to learn more about the mechanisms that control sugar transport into the growing zones of the root. Sugars generated by photosynthesis in leaves are loaded into sieve elements of the phloem and then delivered by phloem into roots (De Schepper et al., 2013). According to pressure flow theory, it is the osmotic differential in phloem sieve elements that drives the bulk flow from source photosynthetic organs to sink organs such as roots. After sugar reaches its destination, it

must be unloaded from phloem sieve elements into surrounding cells. Ross-Elliot et al. (2017) have proposed that after sugar reaches the connecting tissue between conducting phloem and sink tissues, called protophloem, it is unloaded into surrounding cells through funnel plasmodesmata, and then diffuses freely along a concentration gradient into cells of the root tip in a symplastic way. The unloading of sugar into the elongation zone, mitotic zone, and root cap accommodates the high-energy demands of fast-growing regions in roots. Here, we describe the effect of knocking out two of the eight members of the annexin gene family in Arabidopsis, namely *AnnAt1* and *AnnAt2*, on the diffusion of sugars from the unloading zone to root tips, and we discuss how these two annexins play an important mechanistic role in post-phloem transport of sugars into root tips.

2.2 Results

Inhibition of primary root growth in *AnnAt1* and *AnnAt2* knockout mutants is rescued by exogenous sucrose independent of its osmotic effects

In 3-d-old seedlings, all *AnnAt1* and *AnnAt2* knockout mutants (*ann1-2* and *ann1-3*, *ann2-1* and *ann1-2/ann2-1*) had primary root lengths that were significantly shorter compared to wild-type when grown on agar plates containing no sucrose (Fig. 2.1, A and B). However, these knockout mutants had the same hypocotyl lengths compared to that in wild-type (Fig. 2.2). To rule out that the differences in primary root lengths were due to differences in germination, we also measured growth rates in both mutants and wild-type under the same condition (Fig. 1B). Consistent with the root length data, the primary roots of *ann1-2*, *ann1-3*, *ann2-1* and *ann1-2/ann2-1* grew significantly slower compared to that in wild-type.

This inhibition of primary root growth observed in *AnnAt1* and *AnnAt2* knockout mutants was rescued by 2% sucrose (Fig. 1, A and C). This rescue was not due to the osmotic effect of sucrose in agar plates, since equimolar concentrations of mannitol did not rescue the primary root

growth inhibition in *AnnAt1* and *AnnAt2* mutants (Fig. 1, A and D). Similar results were also found in 1% sorbitol and 1% o-methyl-glucose treatments (Fig. 2.3, C and D), indicating that sucrose functioned either as a carbon source or a signal to rescue the primary root growth inhibition in *AnnAt1* and *AnnAt2* knockout mutants. Since sucrose can be catalyzed by invertase into fructose and glucose, we also tested 1% glucose and 1% fructose treatments (Fig. 2.3, A and B). Both glucose and fructose also rescued primary root growth inhibition in *AnnAt1* and *AnnAt2* knockout mutants.

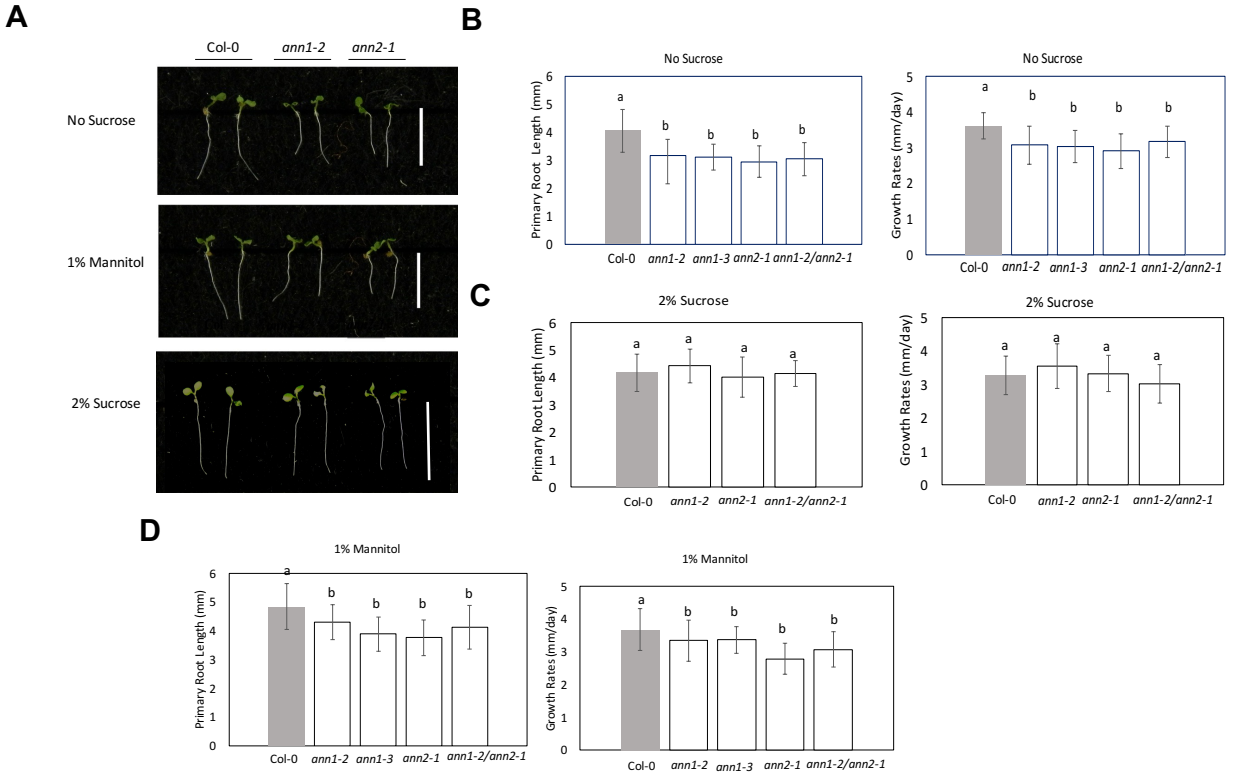

Figure 2.1. Inhibition of primary root growth in *AnnAt1* and *AnnAt2* knockout mutants is rescued by exogenous sucrose independent of its osmotic effects. A, Representative images comparing primary root growth of 3-d-old Arabidopsis wild-type (Col-0), *ann1-2*, *ann1-3*, *ann2-1* and *ann1-2/ann2-1* mutant seedlings grown on vertical MS plates containing either no sucrose, 1% mannitol or 2% sucrose. Bars = 5 mm. B, C and D, Primary root lengths (left graphs) and growth rates (right

graphs) of 3-d-old wild-type (Col-0), *ann1-2*, *ann1-3*, *ann2-1* and *ann1-2/ann2-1* seedlings grown

on vertical MS plates containing either no sucrose (B), 2% sucrose (C) or 1% mannitol (D). Data are means ± SD (n = 16–20) of three independent experiments. Different letters indicate statistically significant differences as evaluated by Student's *t*-test ($P < 0.05$).

Figure 2.1 continued

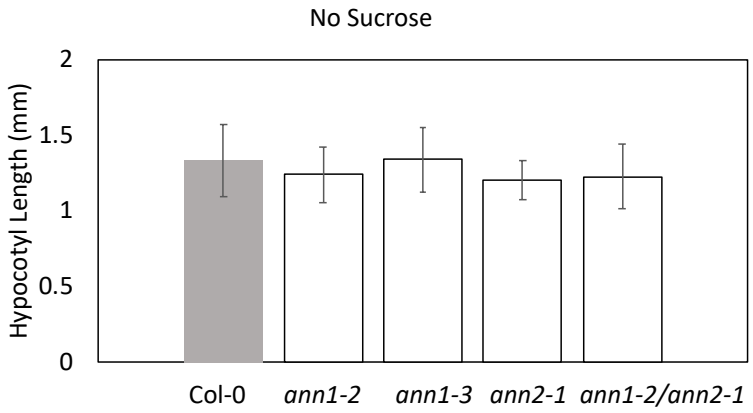

Figure 2.2. Hypocotyl growth of 3-d-old wild type (Col-0) and *AnnAt1* and *AnnAt2* knockout seedlings (*ann1-2*, *ann1-3*, *ann2-1* and *ann1-2/ann2-1* double mutant) grown in the absence of sucrose. Data are means ± SD (n = 15–20) of three independent experiments. No statistically significant difference was observed between between wild type (Col-0) and *ann1-2*, *ann1-3*, *ann2-1*, and *ann1-2/ann2-1* double mutant, evaluated by Student's *t* test ($P < 0.05$).

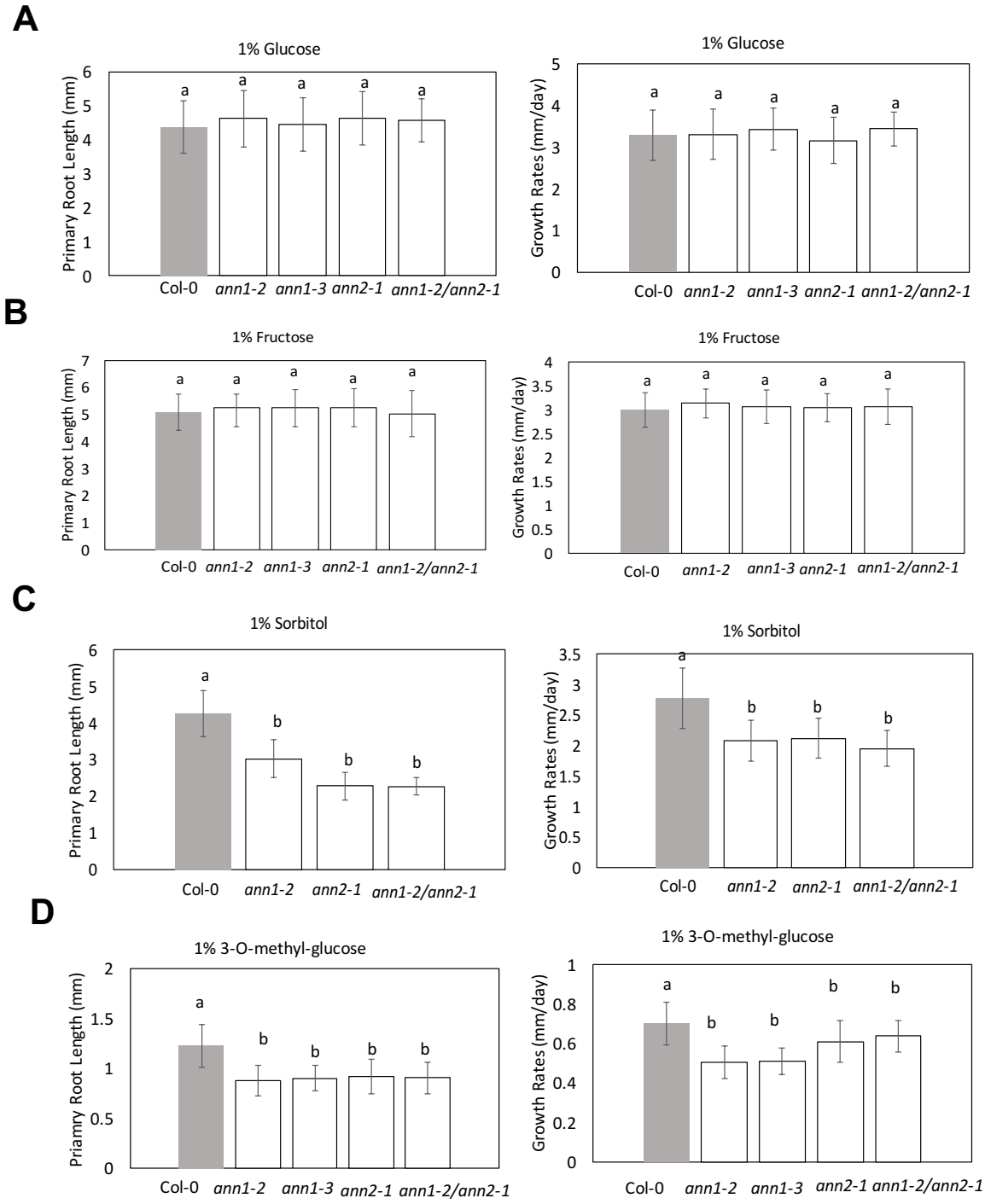

Figure 2.3. Inhibition of primary root growth in *AnnAt1* and *AnnAt2* knockout mutants is rescued by exogenous glucose and fructose independent of their osmotic effects. Comparison of

primary root growth of 3-d-old wild type (Col-0), *ann1-2*, *ann1-3*, *ann2-1* and *ann1-2/ann2-1*

double mutant Arabidopsis seedlings grown on vertical MS plates containing 1% glucose (A), 1% fructose (B), 1% sorbitol (C) and 1% 3-O-methyl-glucose (D). Data are means ± SD (n = 15–20) of three independent experiments. Different letters indicate statistically significant differences between Col-0 and *ann1-2*, *ann1-3*, *ann2-1*, and *ann1-2/ann2-1* double mutant, evaluated by Student's *t* test ($P < 0.05$).

Figure 2.3 continued

Inhibition of root cap development in *AnnAt1* and *AnnAt2* knockout mutants is rescued by exogenous sucrose

As observed by light microscopy of 2 mm segments of root tips from 10 d old seedlings, knocking out *AnnAt1* or *AnnAt2* affected primary root structures of 4-d-old *ann1-2*, *ann1-3*, *ann2-1*, and *ann1-2/ann2-1* seedlings when grown without sucrose. Starch detection by Lugol staining revealed reduced layers of starch-containing columella cells in *ann1-2*, *ann1-3*, *ann2-1* and *ann1-2/ann2-1*, compared to that in wild-type (Fig. 2.4A). In wild-type root caps, there are four layers of columella cells with Lugol-stained starch granules in them, whereas only three layers of columella cells were detected in *AnnAt1* and *AnnAt2* knockout mutants. Also, unlike wild-type roots, the roots of *AnnAt1* and *AnnAt2* mutants had no border-like cells around their root caps. These root cap defects in *ann1-2*, *ann1-3*, *ann2-1* and *ann1-2/ann2-1* were rescued by 1% sucrose (Fig. 2.4B). When grown with 1% sucrose for 4 d, *ann1-2*, *ann1-3*, *ann2-1* and *ann1-2/ann2-1* roots showed four layers of starch-containing columella cells like those in wild-type with border-like cells attached to the root caps.

Because Lugol staining (Fig. 2.4A) revealed similar levels of starch granules in *ann1-2*, *ann2-1* and *ann1-2/ann2-1* when seedlings were grown without sucrose, *ann1-2* was used as a representative line for all *annAt* mutants in subsequent quantification of root-tip starch levels (Fig.

2.5). Consistent with Lugol staining, *ann1-2* displayed significantly lower levels of starch in root tips compared to that in wild-type when grown without sucrose (Fig. 2.5).

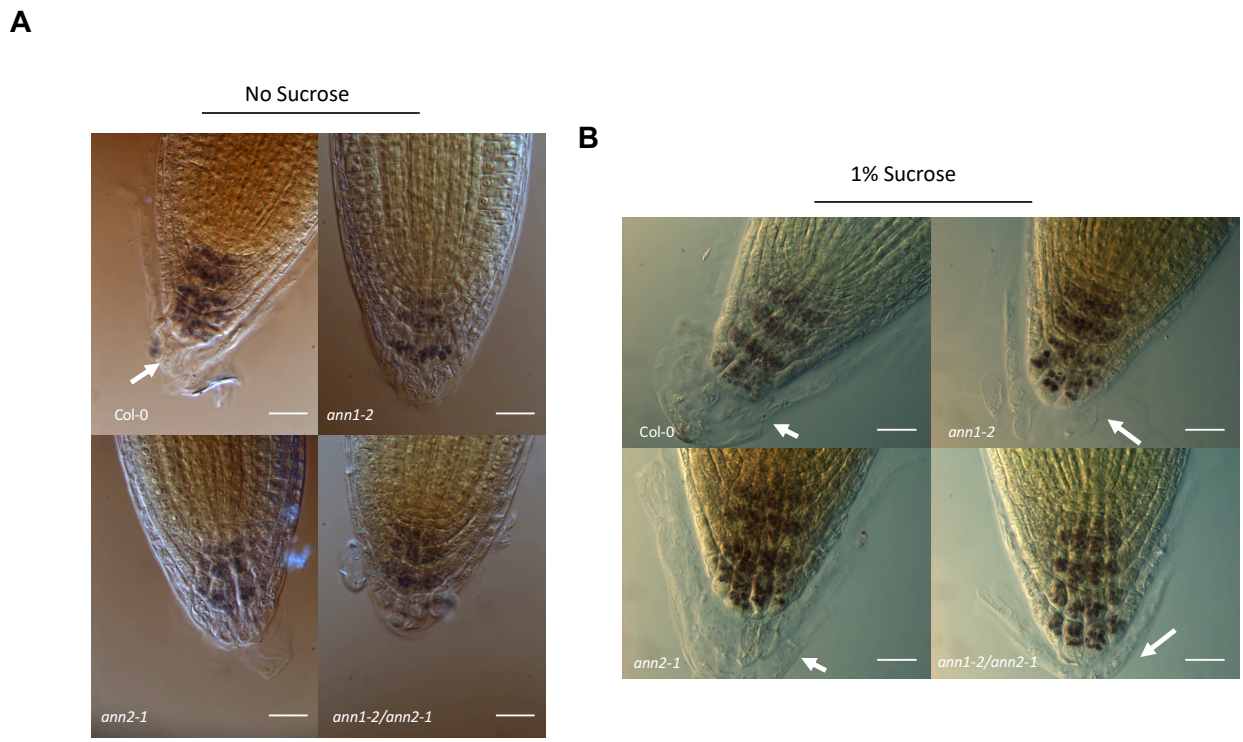

Figure 2.4. *AnnAt1* and *AnnAt2* knockout mutants have root cap developmental defects when grown without sucrose. A and B, Root cap features revealed by Lugol staining of granule starch in columella cells of 4-d-old Arabidopsis wild-type (Col-0), *ann1-2*, *ann2-1* and *ann1-2/ann2-1* seedlings with or without 1% sucrose. Cells with columella identity were detected by staining with Lugol solution. 10–13 seedlings of each set of wild-type (Col-0) and mutant plants were observed. Root tips with stained columella cells of seedlings grown without sucrose (A) and with 1% sucrose (B). White arrow indicates border-like cells. Bars = 10 μm.

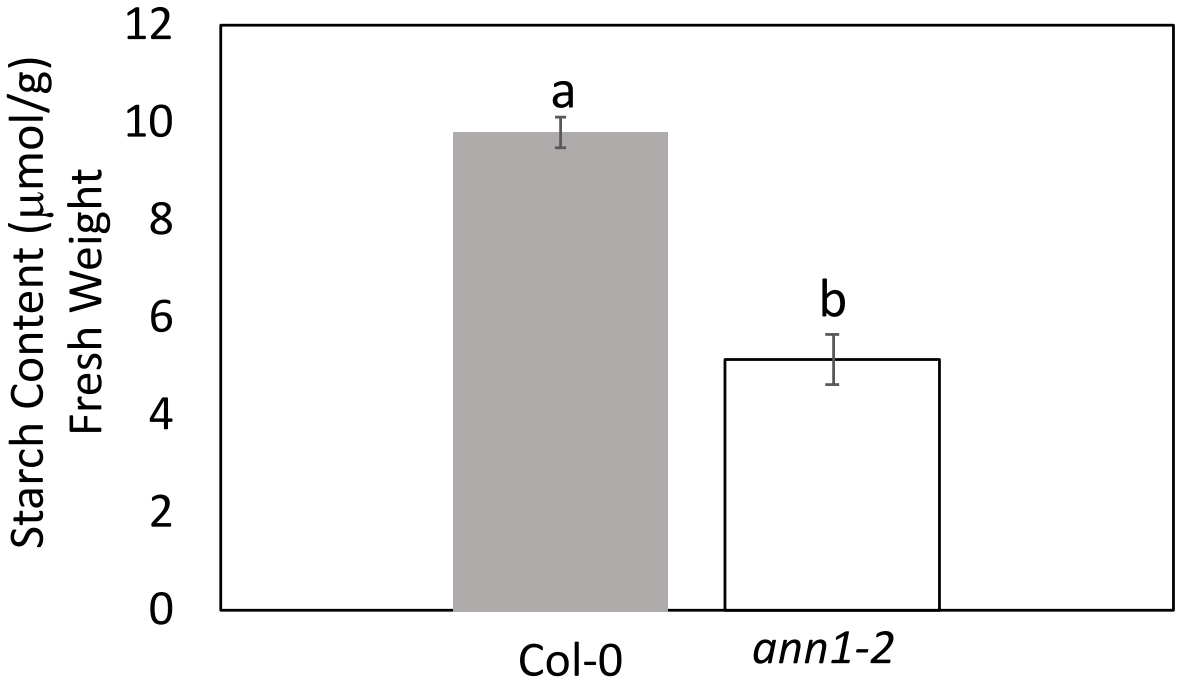

Figure 2.5. *AnnAt1*-mutant root tips contain less starch than wild-type root tips when grown without sucrose. Quantitative analysis of starch content levels in root tips of wild-type (Col-0) and *ann1-2* seedlings grown without sucrose. Starch levels were measured in 2 mm root tips harvested from 10-d-old seedlings grown without sucrose. Data represent means ± SD of two replicates. Different letters indicate statistically significant differences as evaluated by Student's *t*-test ($P < 0.05$).

Expression of photosynthesis-related genes and chlorophyll accumulation is enhanced in *AnnAt1* knockout mutants, dependent on the absence of exogenous sucrose

An RNA-seq analysis comparing the transcript abundance in total mRNA extracted from primary roots of 2-week-old wild-type, *ann1-2* and *ann2-1* grown without sucrose in light revealed

many differentially expressed genes (DEG) in the primary roots of the mutants. After the DEGs were assigned to gene ontology (GO) terms, the GO analysis in *ann1-2* revealed functional enrichment in photosynthesis with seven out of a total of 13 up-regulated GO categories related to photosynthesis (Table 2.1). This indicated significant enhancement in the expression levels of photosynthetic genes in *ann1-2* in the absence of exogenous sucrose.

Table 2.1. GO analysis of *ann1-2* up-regulated genes

GO term	Description	Overlap Count	GO count	Adj. P-value
GO:0030076	light-harvesting complex	15	20	5.0E-07
GO:0016168	chlorophyll binding	18	32	1.9E-05
GO:0015979	photosynthesis	62	188	2.0E-05
GO:0009860	pollen tube growth	74	248	1.5E-04
GO:0009827	plant-type cell wall modification	53	171	8.9E-04
GO:0016114	terpenoid biosynthetic process	16	35	2.0E-03
GO:0006091	generation of precursor metabolites and energy	25	67	2.9E-03
GO:0030599	pectinesterase activity	24	69	1.3E-02
GO:2000122	negative regulation of stomatal complex development	3	3	1.7E-02
GO:0030093	chloroplast photosystem I	3	3	1.7E-02
GO:0009522	photosystem I	4	5	1.7E-02
GO:0009538	photosystem I reaction center	6	11	3.8E-02
GO:0009769	photosynthesis, light harvesting in photosystem II	4	6	4.4E-02

Although Arabidopsis roots partially turn green when exposed to light, the main energy source that facilitates root growth originates in the leaves (Kobayashi et al., 2012). Thus, we decided to assay transcript levels of photosynthetic genes in the cotyledons of 4-d-old *ann1-2* and wild-type seedlings grown without sucrose. We assayed 4-d-old seedlings because evidence shows that the dominant roles of sugar to regulate root growth are probably restricted to the first 5–6 d before leaf development (Kircher and Schopfer, 2012). Among all the genes in photosynthesis-related GO categories, the top five most up-regulated ones were selected to be analyzed by RT-

qPCR. Primer sequences used in RT-qPCR experiments to detect the 4 gene expression are listed in Table 2.2. Results showed that four out of five photosynthetic genes were significantly up-regulated in cotyledons of *ann1-2* seedlings grown without sucrose (Fig. 2.6A). These four genes encode chlorophyll-binding protein 2 (CAB2, LHCB1.1), chlorophyll-binding protein 3 (CAB3, LHCB1.2*)*, and two subunits of photosystem II light harvesting complex (PSII, LHB1B1 and LHB1B2). Gene AT5G28450, which encodes another chlorophyll-binding protein, shows no expression difference between wild type and *ann1-2*.

We also quantified the accumulation of chlorophyll in cotyledons of 4-d-old *ann1-2* and wild-type seedlings grown without sucrose. Consistent with the up-regulation of photosynthetic gene expression, cotyledons of *ann1-2* seedlings showed significantly higher intensity of chlorophyll auto-fluorescence signal than that in wild-type cotyledons, indicating a higher chlorophyll content in cotyledons of *ann1-2* seedlings compared to that in wild-type (Fig. 2.6, B and C).

Table 2.2. Primer sequences of genes tested in qRT-PCR

id	Gene name	Forward 5'-3'	Reverse 5'-3'
AT1G35720	*AnnAt1*	GAAAGTCATCAGGCAAGCAT	GTCCACAACAAGA TAGCTCTC
AT5G65020	*AnnAt2*	GGGTTCTTGTTGAAATCGCT	GAGGAAGCAAGA GCTTACGA
AT2G34420	*LHB1B2*	TGGCTATGTTCTCTATGTTTGG	TCTCTTTCTCTGCT CTCATTCA
AT1G29910	*LHCB1.2*	TGGCTATGTTCTCTATGTTTGG	GGTTCTCTATCGG TCCCTTA
AT2G34430	*LHB1B1*	CCGTGAGCTAGAAGTTATCCA	GTTGCCCAAGTAG TCCAATC
AT1G29920	*LHCB1.1*	TGGCTATGTTCTCTATGTTTGG	GGTTCTCTATCGG TCCCTTA

Exogenous sucrose reversed the enhanced expression of photosynthetic genes and chlorophyll accumulation in cotyledons of *ann1-2* seedlings. When grown with 1% sucrose, *ann1-*

2 cotyledons showed similar photosynthetic gene expression levels as that in wild-type (Fig. 2.6D), and accumulation of chlorophyll in these tissues was also comparable (Fig. 2.6E and F).

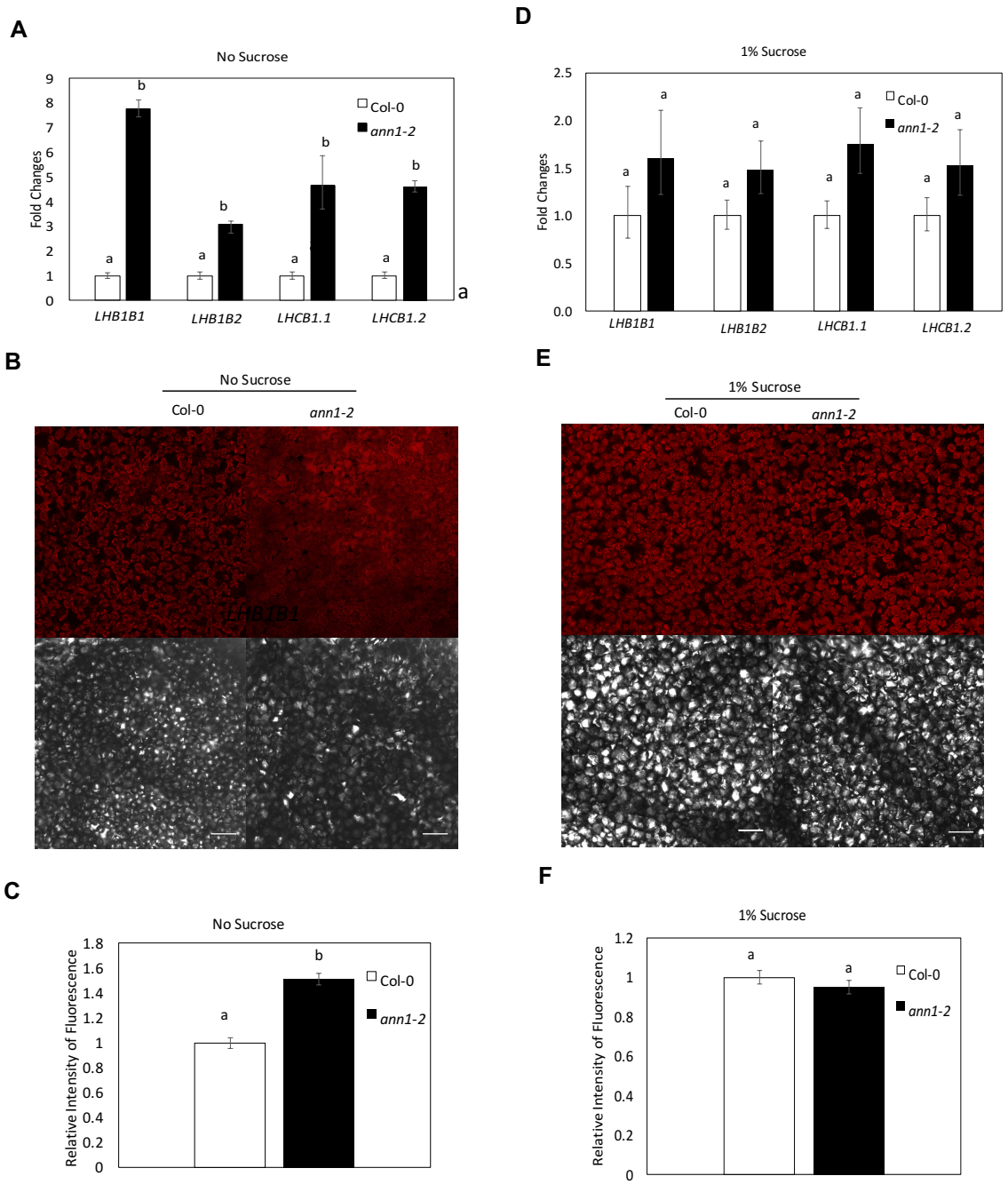

Figure 2.6. Up-regulation of photosynthesis-related gene expression and increased chlorophyll fluorescence in *AnnAt1* knockout mutant is rescued by exogenous sucrose.

A and D, RT-qPCR analysis of gene expression levels in cotyledons of 4-d-old wild-type (Col-0) and *ann1-2* seedlings grown without sucrose (A) or with 1% sucrose (D). Data represent means ± SE (n = 3) of three biological replicates. B and E, Confocal microscopy of chlorophyll fluorescence

in cotyledons of 4-d-old wild-type (Col-0) and *ann1-2* seedlings grown without sucrose (B) or with 1% sucrose (E). Top, chlorophyll fluorescence. Bottom, bright field. 15 seedlings were assayed for each set. Bars = 100 μm. C and F, Quantification of chlorophyll fluorescence relative intensity in cotyledons of 4-d-old wild-type (Col-0) and *ann1-2* seedlings grown without sucrose (C) or with 1% sucrose (F). Values are means ± SE (n = 15) of three independent experiments. Different letters indicate statistically significant differences as evaluated by Student's *t*-test ($P < 0.05$).

Figure 2.6 continued

Knocking-out *AnnAt1* and *AnnAt2* induces accumulation of soluble sugars in primary roots

We quantified neutral soluble sugars in primary roots of *ann1-2*, *ann2-1* and wild-type by GC/MS using primary roots collected from 1-week-old seedlings grown on agar plates containing no sugars (Table 3). Unexpectedly, significantly higher amounts of sugars were detected in both *ann1-2* and *ann2-1* root tissue relative to that in wild-type (Fig. 2.7A), including significantly higher contents of soluble glucose (Fig. 2.7B) and fructose (Fig. 2.7C). Most of the sugar detected was probably in the phloem.

Table 2.3. Quantitative analysis of soluble carbohydrates in primary roots of Col-0, *ann1-2* and *ann2-1* when grown without sucrose			
Glycosyl residue (%, root DW)	Col-0	*ann1-2*	*ann2-1*
Arabinose	1.06 ± 0.281	1.586 ± 0.52	2.267 ± 0.110
Rhamnose	0.794 ± 0.053	1.095 ± 0.371	1.499 ± 0.207
Ribose	0.118 ± 0.022	0.133 ± 0.016	0.285 ± 0.105
Fucose	0.15 ± 0.023	0.2032 ± 0.006	0.295 ± 0.033
Xylose	0.709 ± 0.111	0.931 ± 0.158	1.486 ± 0.33
Mannose	0.209 ± 0.014	0.209 ± 0.107	0.428 ± 0.127
Galactose	3.386 ± 0.379	8.779 ± 0.795	6.959 ± 0.602
Glucose	1.218 ± 0.133	1.916 ± 0.179	2.794 ± 0.391

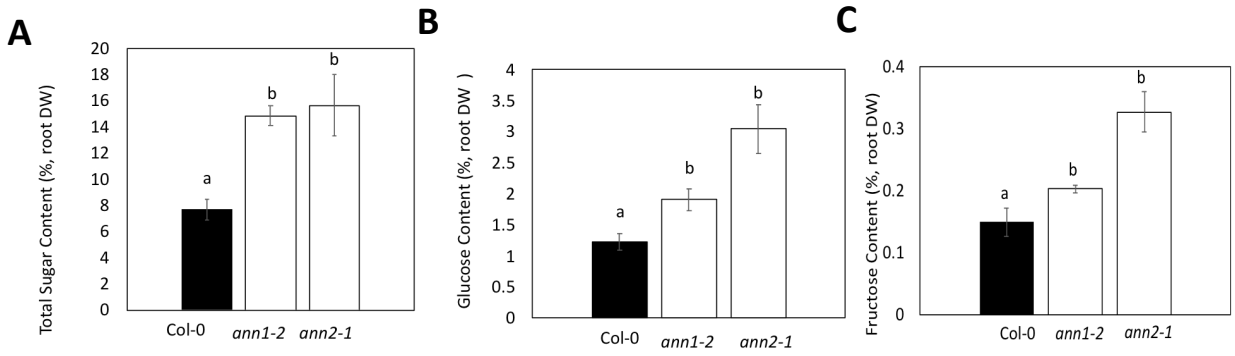

Figure 2.7. *AnnAt1* and *AnnAt2* knockout mutant roots contain significantly more soluble carbohydrates in primary roots when grown without sucrose. A to C, Primary roots were harvested from one-week-old wild type (Col-0), *ann1-2* and *ann2-1* seedlings grown on agar plates containing no sucrose. Soluble sugar contents were analyzed by GC/MS to reveal total sugar (A), glucose (B) and fructose (C) contents. Values are means ± SD of biological triplicates. Different letters indicate statistically significant differences as evaluated by Student's *t*-test ($P < 0.05$).

Knocking-out *AnnAt1* and *AnnAt2* impairs post-phloem transport in root tips

Because 5(6)-Carboxyfluorescein diacetate (CFDA) has been widely used as a phloem-mobile probe (Oparka et al., 1994; Knoblauch et al., 2015), we used CFDA to detect post-phloem transport in wild-type, *ann1-2*, *ann2-1* and the *AnnAt1* overexpressing line *35S:ANN1*. *35S:ANN1* seedlings showed significantly longer roots compared to that in wild-type when grown without sucrose (Supplemental Figure S3). All seedlings were grown without sucrose for 4 d. After applying CFDA on cotyledons for 30 min, the dye was apparent in the two phloem files in primary roots of *ann1-2*, *ann2-1*, *35S:ANN1* and wild-type (Fig. 2.8A), indicating successful loading of CF. Wild-type showed a characteristic transport pattern of CF along root cortex (Fig. 2.8B). However, in *ann1-2* and *ann2-1*, the diffusion of CF in root tips was restricted (Fig. 2.8B).

The post-phloem region has been defined as the region 250 μm behind the root tip (Stadler et al., 2005; Ross-Elliott et al., 2017), so we quantified CF fluorescence in three different areas (transition zone, meristem zone and root cap) of this post-phloem region in *ann1-2*, *ann2-1*, *35S:ANN1* and wild-type roots (Fig. 2.8C). Significantly lower levels of CF fluorescence were detected in both *ann1-2* and *ann2-1* roots compared to that in wild-type when seedlings were grown without sucrose. This significant decrease of CF diffusion was more obvious in root caps, reaching a level more than 50% less compared to that wild-type. In contrast to that in *ann1-2* and *ann2-1*, *35S:ANN1* showed significantly higher levels of CF diffusion in root tips compared to that in wild-type by more than 50% in root caps. Taken together, these results indicate that *ann1-2* and *ann2-1* roots had impaired post-phloem transport; i.e. knocking-out *AnnAt1* and *AnnAt2* restricted the diffusion of sugars from the phloem to the root tip.

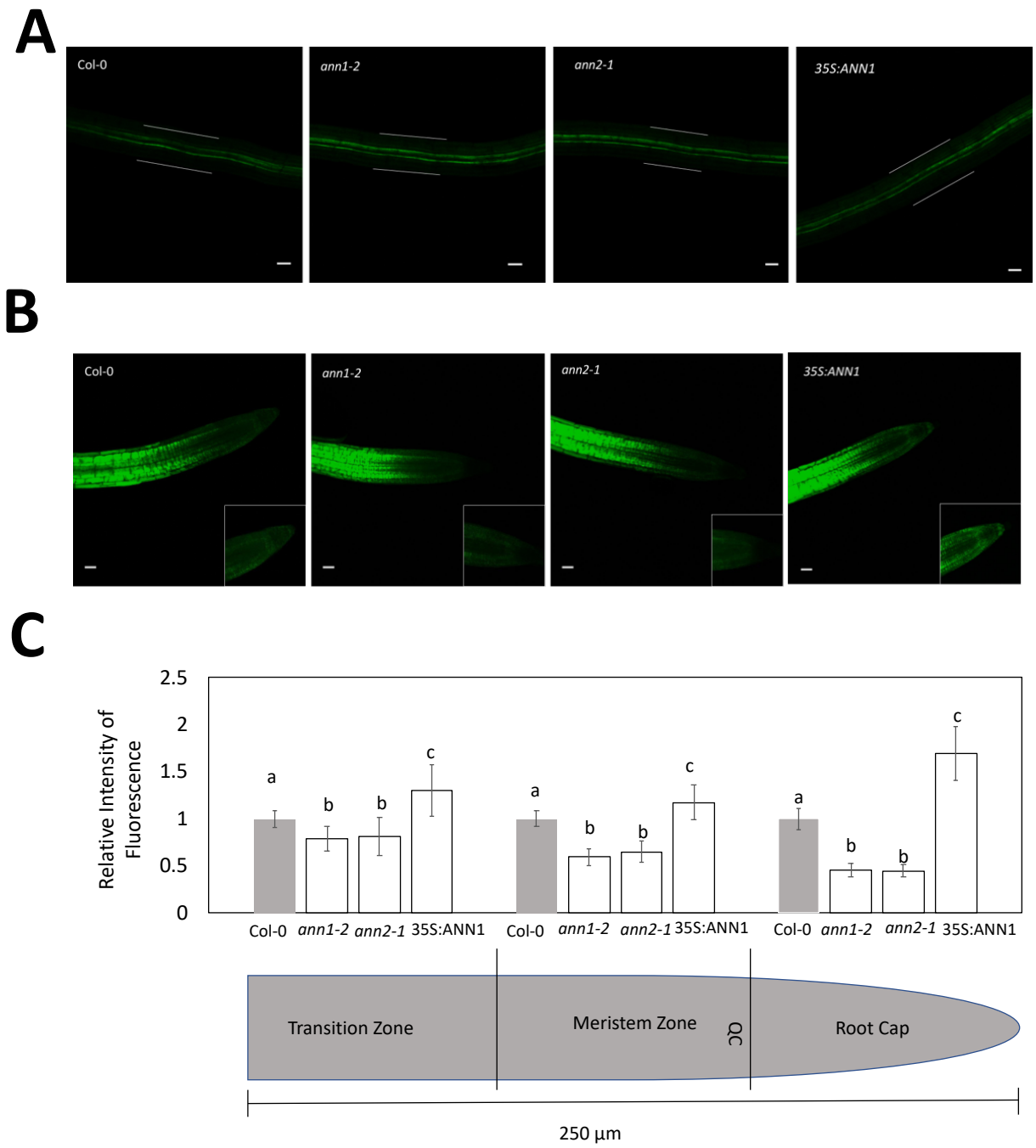

Figure 2.8. ***AnnAt1*** **and** ***AnnAt2*** **knockout mutant roots have restricted diffusion of CF to root tips when grown without sucrose.** A, Representative images showing successful loading of CF in phloem files. White lines indicate edge of root tissue. B, Representative fluorescence micrographs of root tips from wild type (Col-0),

ann1-2, *ann2-1* and *35S:ANN1* (*AnnAt1* overexpression) seedlings grown without sucrose. Bar =

50 μm. C, Quantification of fluorescence levels in different root-tip zones as indicated in wild type (Col-0), *ann1-2*, *ann2-1* and *35S:ANN1* seedlings grown without sucrose. QC, quiescent center. Values are means ± SE ($n \geq 8$; n indicates biological replicates). Different letters indicate statistically significant differences as evaluated by Student's *t*-test ($P < 0.05$).

Figure 2.8 continued

Knocking out *AnnAt1* and *AnnAt2* increased ROS levels and callose accumulation in root tips

Intracellular reactive oxygen species (ROS) regulate plasmodesmata permeability through callose deposition (Benitez-Alfonso et al., 2009; Benitez-Alfonso et al., 2011; Stonebloom et al., 2012). To test whether knocking out *AnnAt1* and *AnnAt2* leads to ROS accumulation in root tips, 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA), a dye that stains hydrogen peroxide (H_2O_2), was applied to 3-d-old seedlings grown without sucrose. Significantly higher levels of stain were detected in root tips of *ann1-2* and *ann2-1* compared to that in wild-type when grown without sucrose (Fig. 2.9A and B). Aniline blue was used to detect callose accumulation in root tips of 3-d-old wild-type, *ann1-2* and *ann2-1* seedlings grown without sucrose, which revealed significantly higher levels of callose deposition in *ann1-2* and *ann2-1* compared to that in wild-type (Fig. 2.9C and D). The blue lines in this Figure indicate PD colocalized together. Due to the limitation of confocal resolution (230 nm), single PDs cannot be detected, but this pattern of callose deposition is standardly accepted as PD enrichment [add reference here].

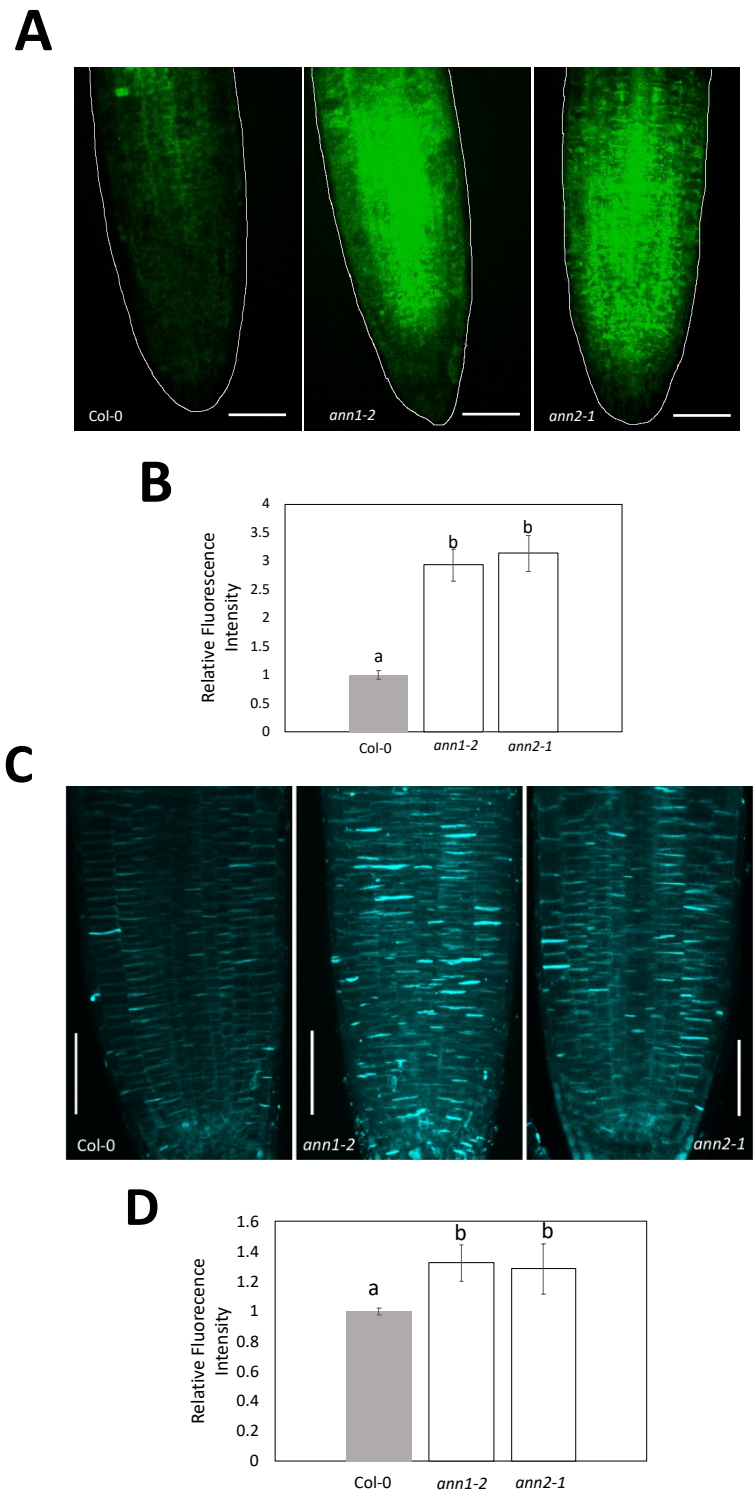

Figure 2.9: continued next page

Figure 2.9. *AnnAt1* and *AnnAt2* knockout mutant roots show increased levels of ROS and callose in root tips when grown without sucrose. A, Representative confocal microscopy images of H_2O_2 levels in root tips as detected by H_2DCFDA staining of wild-type (Col-0), *ann1-2* and *ann2-1* seedlings grown without sucrose for 3 d. B, Quantification of relative fluorescence intensity indicating H_2O_2 levels in root tips of 3-d-old wild type (Col-0), *ann1-2* and *ann2-1* mutant seedlings grown without sucrose. C, Representative confocal microscopy images of callose levels in roots tips as detected by aniline blue staining of wild-type (Col-0), *ann1-2* and *ann2-1* seedlings grown without sucrose for 3 d. D, Quantification of relative fluorescence intensity indicating callose deposition in root tips of 3-d-old wild-type (Col-0), *ann1-2* and *ann2-1* seedlings grown without sucrose. B and D, Results were consistent in three independent experiments. Values are means ± SE ($n \geq 8$). Different letters indicate statistically significant differences as evaluated by Student's *t*-test ($P < 0.05$). Bar = 50 μm.

2.3 Discussion

AnnAt1 and *AnnAt2* knock-out seedlings show sucrose-dependent phenotypes

The observation that significantly higher contents of soluble sugar were detected by GC/MS analysis in primary roots of *ann1-2* and *ann2-1* compared to those of wild-type when grown together without sucrose indicated there was successful phloem transport of sugar from shoots to roots in the knock-out mutants, yet the mutants had obvious growth and columella cell defects. The fact that these defects were rescued by exogenous sucrose independent of its osmotic effects suggested the possibility that *AnnAt1* and *AnnAt2* helped to regulate the post-phloem transport of sucrose into root tips. Although successful unloading was detected in both the *AnnAt1* knockout mutant *ann1-2* and the *AnnAt2* knockout mutant *ann2-1*, the diffusion of CFDA into root tips was impaired in both *ann1-2* and *ann2-1*, indicating these mutants had restricted sugar

diffusion in fast-growing regions from the unloading zone to root tips. These results are consistent with the conclusion that compromised expression of *AnnA1* and *AnnAt2* leads to impaired diffusion of sugars in the fast-growing root apex, and this, in turn, inhibits primary root growth. However, further studies quantifying carbohydrate content only in the cap region after laser micro-dissection are required to confirm this conclusion.

Sugar in regulation of photosynthesis

The regulatory roles of sugar in photosynthetic gene transcripts and photosynthesis capacity have been widely documented (Koch, 1996; Pego et al., 2000). Sugar starvation in plants activates photosynthesis, whereas high sugar levels inhibit photosynthesis (Yu et al., 2015; Sami et al., 2016). Sugar represses the transcriptional activity of photosynthetic gene promoters transiently expressed in maize mesophyll protoplasts (Sheen, 1990). Sugar accumulation in leaves represses photosynthetic gene expression and photosynthesis (Pego et al., 2000). Roots are typical sink organs. Sugar demands in young roots promote photosynthesis in leaves. For example, overexpression of the *HIGHER YIELD RICE* (*HYR*) gene encoding an APETALA2/ETHYLENE RESPONSIVE FACTOR (AP2/ERF) transcription factor leads to increased root length, branching, and strength, which in turn promotes photosynthesis in leaves (Ambavaram et al., 2014). However, the mechanism of how plants monitor sugar levels in roots remains an active area of investigation. Here we hypothesize that root tips are sugar-sensing tissues and their sugar status can affect photosynthesis capacity in leaves.

As noted above, our results revealed sugar starvation in the actively growing root apex of the *ann1-2* and *ann2-1* knockout mutants by using CF as a phloem mobile probe. Evidence that this apical sugar starvation could have led to an up-regulation of photosynthesis was indicated by the increased expression of genes encoding CAB2 (LHCB1.1), CAB3 (LHCB 1.2) and photosystem II subunits (LHB1B1 and LHB1B2), along with increased chlorophyll contents in

ann1-2. The enhancement of photosynthesis in *ann1-2* was most likely the change that resulted in the elevated sugar contents observed in primary roots of *ann1-2*. However, because these accumulated sugars couldn't reach the root apex due to the defective post-phloem transport of sugars in *ann1-2*, they failed to rescue primary root growth defects and to reverse the enhancement of photosynthesis. Assuming that sugars accumulate in the phloem and unloading zone without sufficient diffusion into the post-phloem region, as suggested by the CF data in Fig. 2.9, this transport defect would lead to sugar starvation in root tips. To the extent that the post-phloem zone is the real sugar-sensing area of roots, then, because roots can communicate their nutrient status to shoots and induce responses in them (Ko and Helariutta, 2017), the stress of sugar starvation in root tips could initiate a root-to-shoot signal, just as salt stress in roots promote the propagation of Ca^{2+} waves from roots to shoots (Choi et al., 2014; Evans et al., 2016). Plausibly, such a signal could help explain how sugar-deprived root tips could induce the upregulation of photosynthetic genes in leaves.

Roots efficiently take up sucrose and glucose from the media (Chaudhuri et al., 2008), and consistently, exogenously supplied sugars in the medium successfully rescued primary root growth defects and reversed photosynthesis enhancement in *ann1-2*. These results favor the conclusion that AnnAt1 helps control root sugar status, which would result in potential feedback regulation of photosynthesis. Knocking out *AnnAt1* may result in sugar starvation in roots, which could serve as a signal for plants to up-regulate photosynthesis.

Post-phloem transport of sugar to root tips

Apical sugar concentration in root tips has been considered to play the determinant role in regulating root growth and development (Freixes et al., 2002). The diffusion of CF in root tips of *ann1-2* and *ann2-1* was restricted, whereas the *AnnAt1* ovexpressing line *35S:ANN1* showed promoted diffusion of CF. We interpreted these results to indicate that knocking-out *AnnAt1* and

AnnAt2 restricted symplastic diffusion in post-phloem transport from the unloading zone to root tips, and that this restriction impaired primary root growth along with root cap development.

Sugar diffuses freely along concentration gradient through plasmodesmata in the root apex. It is now widely accepted that plasmodesmata are dynamic channels whose permeability is highly regulated during plant growth and development (Sager and Lee, 2014). Callose has been found deposited in the neck region of plasmodesmata and can restrict the size exclusion limit of plasmodesmata (Luna et al., 2011; Zavaliev et al., 2011). ROS accumulation can increase callose accumulation, which may result in reduced plasmodesmatal transport (Benitez-Alfonso et al., 2009, 2011; Stonebloom et al., 2012).

AnnAt1 has antioxidant activity (Gidrol et al., 1996), and this activity may help explain the results of prior studies that documented a link between *AnnAt1* suppression and an increase in ROS levels. For example, the knockout of *AnnAt1* results in the accumulation of higher levels of ROS in leaves compared to that in wild-type (Konopka-Postupolska et al., 2009). Similarly, our results show that knocking out *AnnAt1* and *AnnAt2* leads to hyperaccumulation of ROS in root tips, and, like earlier studies, associates this change with an elevated accumulation of callose. Assuming that some of this accumulated callose is deposited in plasmodesmata, it would restrict plasmodesmatal sugar transport, and thus help explain why post-phloem transport of sugar to root tips is inhibited in mutants.

In conclusion, our results provide data linking AnnAt1 and AnnAt2 to the regulation of primary root growth and development. This study expands our understanding of the function of annexins in plants to include an important role in the post-phloem transport of sugars to the root tip, which, in turn, indirectly impacts photosynthetic rates in cotyledons.

2.4 Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana Colombia (Col-0) ecotype was used as the wild-type in all experiments. *AnnAt1* (At1g35720) and *AnnAt2* (At5g65020) are studied. Annexin T-DNA insertion mutants including *ann1-2*, *ann1-3*, *ann2-1*, *ann1-2/ann2-1* and *35S:ANN1* were used in this study (Wang et al., 2015). All seeds were surface sterilized by 75% (v/v) ethanol for 1 min and 20% bleach (v/v) for 10 min. After washing 5 times with sterile, deionized water, seeds were sowed on agar plates containing either no sucrose, 2% (w/v) sucrose or 1% (w/v) mannitol. Seeds on agar plates were stratified in darkness at 4°C for 3 d. Stratified plates were placed vertically in a growth chamber (Percival AR-66 L; light intensity of 275 /μmol m^{-2} s^{-1}, humidity of approximately 80%, 20°C) in continuous light or in darkness. Unless otherwise noted, chemicals were reagent grade from Sigma-Aldridge Co. (St. Louis, MO).

Lugol staining of columella cells

Seedlings grown with 1% sucrose or without sucrose were submerged in a root cap fixative solution (5% (w/v) formaldehyde, 5% (v/v) acetic acid and 25% (v/v) ethanol) for 24 h. Lugol staining of root caps was done as described by Hong et al. (Hong et al., 2015). 10–13 seedlings were imaged for each genotype with a DIC optics on a **Nikon 90i Stereology microscope**, using a 60 × objective.

Starch quantification

Starch levels in 2mm root tips of 10-d-old wild-type and *ann1-2* seedlings grown without sucrose were assayed in ethanol extracts using methods described by Bergmeyer (1984), Barratt et al. (2009), and Divya et al. (2010).

RNA extraction

Trizol reagent was used in all RNA extraction experiments following the manufacturer's protocol.

RNA-seq analysis

To elucidate the mechanisms of AnnAt1 and AnnAt2 in regulation of primary root growth, we performed a comparative RNA sequencing (RNA-seq) using total RNA from primary roots excised from 2-week-old wild-type, ann1-2 and ann2-1 seedlings grown without sucrose in light. Total RNA extracted from primary roots of 2-week-old wild-type, ann1-2 and ann2-1 seedlings grown without sucrose was submitted to the Genome Sequencing and Analysis Facility (GSAF) at the University of Texas at Austin. Both library construction and sequencing were performed by GSAF. After filtering low-quality reads and removing adaptor contamination from the raw data of Illumina reads, the following steps were as described by Van Verk et al. (2013) to detect differential expression in ann1-2 and ann2-1 compared to Col-0. A cutoff value of a greater than 2-fold change in expression was used to select differentially expressed genes in ann1-2 and ann2-1.

To further investigate the biological functions of the differential expressed genes (DEG) in both ann1-2 and ann2-1, we subsequently assigned the DEGs to gene ontology (GO) terms. TAIR10 was downloaded for gene function descriptions and gene ontology (GO) annotations. Ture-path rule was applied in functional enrichment analysis. A gene annotated with a particular GO term was also annotated with all its parents. To avoid very generic, non-informative terms for analysis, only terms annotating 500 or fewer genes were retained. Genes annotated with a given specific GO term were considered as a gene set. All the gene sets were tested for the statistical significance of enrichment among themselves using the cumulative hypergeometric test. Then a Benjamini-Hochberg false discovery rate (FDR) (Benjamini and Hochberg, 1995) was also calculated. A FDR threshold of 0.05 was used for significance.

RT-qPCR analysis

cDNA was transcribed from RNA by the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher) after DNase I digestion. 10 μg cDNA was used for each sample. RT-qPCR was performed on QuantStudio platform (Applied Biosystems) with PowerUp Sybr Green Master Mix (ThermoFisher). Transcripts of *cab2* (AT1G29920), *cab3* (AT1G29910), *LHB1B1* (AT2G34430), *LHB1B2* (AT2G34420), *AnnAt1* (AT1G35720) and *AnnAt2* (AT5G65020) under different conditions were studied. Specificity of gene amplifications were confirmed by melting curves. For each condition tested, three biological replicates for both wild-type and *ann1-2* with three technical replicates for each biological replicate were used. $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to calculate fold changes. For significant difference between each condition, either one-way ANOVA for three conditions or Student's *t*-test for two conditions was used.

Detection of chlorophyll autofluorescence

Cotyledons of 4-d-old intact *ann1-2* and wild-type seedlings grown either with 1% sucrose or without sucrose were examined. Chlorophyll autofluorescence 660–701 nm was detected with an excitation wavelength of 488 nm. 15 seedlings were examined for *ann1-2* and wild-type .

GC/MS analysis

Wild-type, ann1-2 and ann2-1 seedlings were grown in continuous light on agar plates containing no sucrose for a week. Primary roots were collected and washed in sterile water twice. After freeze drying for 2 d, samples were submitted to complex carbohydrate research center at the University of Georgia for GC/MS analysis. Triplicate experiments were carried out.

CFDA application

CFDA stock was prepared as 6 mg/ml in acetone and kept at -80°C. A dilution of 1:20 in sterile water was used for application. Wild-type, *ann1-2* and *ann2-1* seedlings were grown on agar plates containing no sucrose for 4 d. A cotyledon was grazed by fine tweezers to allow 1µl CFDA to penetrate. All samples were excited by 488 nm laser. Fluorescence 500–566nm was monitored.

ROS detection

3-d-old wild-type, *ann1-2* and *ann2-1* seedlings grown without sucrose were used. Whole seedlings were incubated in the buffer (30 mM KCl and 10 mM MES-KOH, pH 6.15) with 50 µM H2DCFDA for 1 h in darkness at room temperature. Extra dye was washed three times by the buffer before confocal microscopy. All samples were excited by 488 nm laser. Fluorescence between 517–527 nm was collected.

Aniline blue staining

3-d-old wild-type, *ann1-2* and *ann2-1* seedlings grown without sucrose were stained by aniline blue (Biosupplies, AU) as described by Ross-Elliott et al. (2017).

Confocal microscopy

Confocal Imaging was done by a confocal laser scanning microscope (Zesis LSM 710) in a set manner, starting with the same image acquisition settings and data processing for all experiments. Fluorescent signals in all samples were recorded in Z-stacks. Z-project in Fiji was used to flatten z-stacks either with average intensity for better quantification accuracy, or with maximum intensity for better focus in representative images. Relative fold change of fluorescent intensity was calculated by division of the mean fluorescent intensity in wild-type. Student's *t*-test

was used to calculate significant difference between samples. Fluorescence was quantified in a way to avoid size difference.

Chapter 3

Transmission electron microcopy and RNA-seq analyses reveal additional changes induced in Arabidopsis by suppressing AnnAt1 and AnnAt2 expression

3.1 Introduction

Chapter 2 focused narrowly on the effects of knocking out *AtAnn1* and *AtAnn2* on sugar transport in the post-phloem zone of root tips in Arabidopsis. Here, to document additional significant changes induced in the annexin mutants, we present and discuss two other analyses we completed: ultrastructural changes observed in the plasmodesmata by transmission electron microscopy, and the full RNAseq analysis of the transcriptomic changes induced in the annexin mutants beyond those discussed in Chapter 2.

Research described in Chapter 2 revealed restrictions of symplastic sucrose transport in *ann1* and *ann2* roots, and we proposed these restrictions were potentially due in part to ROS-induced callose deposition on PD. However, that work did not evaluate whether knocking out *AnnAt1* and *AnnAt2* actually alters PD structure in the root post-phloem region. In this chapter I address this question by presenting data on PD structures in the root post-phloem region of *ann1* and *ann2* observed under transmission electron microscope. To better appreciate the significance of these ultrastructural studies, more background information on the structure and regulation of PD are addresses.

Plasmodesmata are cytosolic bridges that connect adjacent cells and that allow nutrients and macromolecules, including transcription factors and all kinds of RNA, to pass through. The plasmodesmatal movement of small molecules and macromolecules plays critical roles in plant development (Benitez-Alfonso et al., 2013; Gallagher et al., 2014) and in how plants adapt themselves to environmental stimuli (Brunkard and Zambryski, 2017; Ganusova and Burch-Smith, 2019). For example, the transcription factor SHR, which is first produced in stele cells, moves to the endodermis through PD to activate the transcription factor SCR. SCR then functions together with SHR to activate the transcription of the micro RNAs, *MIR165a* and *MIR166b*. The movement

of miR165/166 through PD from endodermis to vascular cylinder regulates the level of PHB transcripts and thereby specifies proper xylem development (Carlsbecker et al., 2010; Miyashima et al., 2011; Vaten et al., 2011; Furuta et al., 2012).

Another well-described example of the critical role of transcription factor movement in the control of plant development is the transport of WUSCHEL (WUS). This transcription factor is first synthesized in the organizing center of shoot apical meristems (SAM), then moves through PD to the central zone where it activates its negative regulator *CLAVATA3* to restrict the accumulation of WUS in the central zone. The restriction of WUS accumulation in the central zone of SAM is required for plants to maintain a constant number of stem cells in the SAM (Brand et al., 2000; Yadav et al., 2011).

The permeability of PD can be regulated. Extensive studies of plant viral systematic infection have revealed a group of movement proteins (MP) localized on plant PD (Lucas and Gilbertson, 1994; Lazarowitz and Beachy, 1999). Tobacco Mosaic Virus (TMV) MPs have shown to dilate PD and to bind dsDNA/ssRNA to facilitate viral infection in mesophyll cells. Constitutive expression of the NS_M viral movement protein (MP) of tomato leads to a basal defense response from plant by deposition of callose on NS_M-targeted mesophyll PD (Rinne et al., 2005).

The biogenesis of PD has been well described (Brunkard and Zambryski, 2017). Primary PD are formed in newly synthesized cell walls during cell division, while secondary PD are formed on existing cell walls to connect adjacent cell files. Primary PD and secondary PD share the same structure, which consists of an external membrane that is continuous with the cell membrane, a compressed strand of endoplasmic reticulum called desmotubule, and a cytosolic sleeve through which small molecules pass (Brunkard et al., 2013; Kragler, 2013). A main question on plasmodesmatal movement is whether cargoes have to be below a specific size. Among the first transcription factors shown to move through PD was KN1, a 41 kDa protein (Lucas et al. 1995). Since KN1, a large number of intercellular trafficking transcription factors beyond the size exclusion limit (SEL) has been discovered (Lucas et al., 1995; Wu and Gallagher, 2011). In addition to passive diffusion through the cytoplasmic sleeve of small soluble proteins (<40kDa)

(Gallagher et al., 2014), proteins unfold when they pass through PD and refold afterwards (Xu et al., 2011; Gallagher et al., 2014). Another method of plasmodesmatal movement of proteins beyond the SEL is to interact with PD to increase the SEL of the cytoplasmic sleeve and promote their own intercellular movement (Crawford and Zambryski, 2000, 2001). Also, small membrane proteins can pass through the desmotubule by an unrevealed mechanism.

Although plasmodesmatal movement is passive, the gating of PD can be regulated by the homeostasis of callose synthesis and degradation on PD (Amsbury et al., 2017; Wu et al., 2018; Benitez-Alfonso, 2019). The synthesis and deposition of callose on PD are controlled by multi-subunit callose synthases (CalS) complexes (CalSC). The clear composition of CalSC is still under study. Until now, the revealed protein subunits are: CalS or glucan-synthase like (GSL) to synthesize callose, a sucrose synthase (SuSy) to convert sucrose to UDP-glucose, a UDP-glucose to transfer the substrate to CalS, and a GTPase to regulate the activity of CalSC. There are 12 genes that encode CalS in *Arabidopsis thaliana*, where the expression of this complex is regulated by developmental stages and multiple environmental signals. For example, a superoxide burst induced in plants by salicylic acid by pathogen infections may promote CalS activity and thereby restrict plasmodesmatal permeability (Cheval and Faulkner, 2018). CalS7 is specifically expressed in phloem, while CalS3 is found in late flower stages (Klepikova et al., 2016). The degradation of callose is carried out by PD-localized (1,3)-β-glucanases (PDBG). Until now, four PDBGs have been described in Arabidopsis: PDBG1 (At3g13560), PDBG2 (At2g01630), PDBG3 (At1g66250) and AtBG_pap (At5g42100). Orthologs of these genes in *Populus* are differentially regulated by GA and photoperiod (Rinne et al., 2011). Another signal regulating callose deposition on PD is ROS (Benitez-Alfonso et al., 2011) and as shown in chapter 2 there are differences in ROS levels depending on the expression level of *AnnAt1* and *AnnAt2*, although the mechanism by which it promotes the deposition is still undiscovered.

Regarding our RNA-seq analyses, our data are the first to document the global transcriptomic changes that result from knocking out *AnnAt1* and *AnnAt2*.

3.2 Results

Transmission Electoral Microscope (TEM) results reveal altered structures of PD in *ann1* and *ann2* roots

Symplastic diffusion of CFDA in *ann1* and *ann2* roots is restricted, indicating defective diffusion of sugar in *ann1* and *ann2* roots, and Figure 3.1 reveals that the structure of PD in mutant roots is altered, which may help account for the defective sugar transport through PD.

In Col-0 wild type, 5 out of 5 PD are observed as simple PD on phloem pole pericycle cells. However, 3 out of 5 PD are funnel shaped PD in *ann1*. In *ann2*, 5 out 5 PD lack desmotubules or have abnormally shaped desmotubules, whereas all 5 PD in Col-0 wild type have normal desmotubules.

Due to the lack of information on PDs in the meristem, whether the quantity and type of PD are consistent across the meristem remains unknown. Evidence reveals there are differences in PD type and quantity in different cell types associated with the phloem (Ross-Elliot et al, 2017). It would be possible that PD may differ between cell layers in meristem.

Figure 3.1. Representative TEM images of PD in roots of Col-0 wild type, *ann1* and *ann2*. Seedlings were grown on agar without sucrose for 3 days. Red circle, PD. Red arrow, desmotubule in PD. Scale bar, 200 nm. Note that because the fixation method used is chemical,

and chemicals penetrate cells slowly at room temperature, cell structures change during the penetration process, thus the images here do not reflect actual PD and wall dimensions.

Figure 3.1 continued

RNA-seq analyses reveal significant changes in the abundance of multiple transcripts in the annexin mutants

Using the RNA extracted from primary roots of two-week old WT and *ann1* and *ann2* grown without sucrose, we analyzed transcript abundance differences between WT and mutant roots. Using a cut-off for significance of 2-fold, a total of 7956 genes changed abundance in *ann1*, 4866 upregulated, and 3090 down regulated (Table 3.1). In *ann2*, the number of genes up-regulated was 2589, while the number of genes down-regulated was 3509. To learn whether the genes that were differentially-expressed by the suppression of *AnnAt1* and *AnnAt2* preferentially functioned in certain biological processes or molecular activities, gene ontology (GO) analysis was carried out (Table 2.1, Table 3.2, Table 3.3 and Table 3.4). Presented here are only those GO categories with an Adjusted p-value of less than 0.01. In *ann1*, 73 GO categories were down-regulated (Table 3.2). The main down-regulated categories were related to cell division (8) and chromatin modification (10).

In *ann2*, 13 GO categories were up-regulated (Table 3.3), while 34 were down-regulated (Table 3.4). 11 out of 34 down-regulated categories were immune response-related.

RNA-seq data will be statistically evaluated after completing RNA-seq analyses of two additional biological replicates for *ann1*, *ann2* and wild type. Detailed descriptions and full lists of genes with significant changes will be released at that time.

Table 3.1. number of differentially expressed genes in *ann1* and *ann2*

	Number of genes upregulated	Number of genes down-regulated
ann1	4866	3090
ann2	2589	3509

Table 3.2 GO analysis of *ann1* down-regulated genes

GO term	Description	Overlap Count	GO count	Adj. P-value
GO:0051567	histone H3-K9 methylation	67	179	3.99E-18
GO:0006275	regulation of DNA replication	48	110	3.43E-16
GO:0006306	DNA methylation	61	168	9.25E-16
GO:0000911	cytokinesis by cell plate formation	63	189	3.50E-14
GO:0008283	cell proliferation	57	164	9.39E-14
GO:0006260	DNA replication	40	94	4.41E-13
GO:0010200	response to chitin	102	421	3.99E-12
GO:0051726	regulation of cell cycle	50	144	4.57E-12
GO:0006270	DNA replication initiation	31	65	7.63E-12
GO:0010106	cellular response to iron ion starvation	42	116	7.27E-11
GO:0000041	transition metal ion transport	41	114	1.67E-10
GO:0010389	regulation of G2/M transition of mitotic cell cycle	28	60	1.92E-10
GO:0006826	iron ion transport	40	117	1.83E-09
GO:0006342	chromatin silencing	41	123	2.68E-09
GO:0016458	gene silencing	27	62	3.19E-09
GO:0044036	cell wall macromolecule metabolic process	31	80	5.82E-09
GO:0034968	histone lysine methylation	34	94	7.71E-09
GO:0010089	xylem development	32	87	1.48E-08
GO:0052542	defense response by callose deposition	22	47	2.50E-08
GO:0009611	response to wounding	74	327	2.31E-07
GO:0016572	histone phosphorylation	24	61	3.50E-07
GO:0010075	regulation of meristem growth	43	154	4.73E-07
GO:0000226	microtubule cytoskeleton organization	40	142	1.08E-06

Table 3.2 continued

GO:0010014	meristem initiation	37	127	1.24E-06
GO:0031047	gene silencing by RNA	31	97	1.26E-06
GO:0051225	spindle assembly	19	44	1.49E-06
GO:0006952	defense response	77	368	4.25E-06
GO:0009693	ethylene biosynthetic process	34	117	4.32E-06
GO:0035556	intracellular signal transduction	38	138	4.33E-06
GO:0016132	brassinosteroid biosynthetic process	34	118	5.42E-06
GO:0006261	DNA-dependent DNA replication	30	99	7.52E-06
GO:0003777	microtubule motor activity	23	66	9.01E-06
GO:0009813	flavonoid biosynthetic process	22	63	1.47E-05
GO:0009718	anthocyanin-containing compound biosynthetic process	19	50	1.65E-05
GO:0016570	histone modification	21	60	2.40E-05
GO:0042546	cell wall biogenesis	21	61	3.28E-05
GO:0009855	determination of bilateral symmetry	33	122	3.88E-05
GO:0048589	developmental growth	18	50	7.48E-05
GO:0009612	response to mechanical stimulus	19	56	0.00011795
GO:0006346	methylation-dependent chromatin silencing	31	117	0.00012147
GO:0042127	regulation of cell proliferation	16	43	0.00013841
GO:0048449	floral organ formation	22	74	0.00029764
GO:0009963	positive regulation of flavonoid biosynthetic process	27	101	0.00036332
GO:0000280	nuclear division	15	42	0.0004364
GO:0007020	microtubule nucleation	19	62	0.00061116
GO:0031048	chromatin silencing by small RNA	29	115	0.0006405
GO:0007231	osmosensory signaling pathway	4	4	0.00064353
GO:0033500	carbohydrate homeostasis	4	4	0.00064353
GO:0009909	regulation of flower development	58	292	0.00070585
GO:0006268	DNA unwinding involved in replication	6	9	0.00075799
GO:0048527	lateral root development	22	79	0.0009171
GO:0015706	nitrate transport	44	206	0.00093339
GO:0010167	response to nitrate	42	195	0.00110024
GO:0048364	root development	31	131	0.0013842
GO:0048869	cellular developmental process	5	7	0.00156182
GO:0010054	trichoblast differentiation	15	47	0.00192406
GO:0080167	response to karrikin	30	128	0.00213173
GO:0010413	glucuronoxylan metabolic process	38	176	0.00222388
GO:0010224	response to UV-B	25	100	0.00229858

Table 3.2 continued

GO:0045492	xylan biosynthetic process	38	177	0.00252844
GO:0009944	polarity specification of adaxial/abaxial axis	21	79	0.00269956
GO:0030414	peptidase inhibitor activity	4	5	0.00292794
GO:0009653	anatomical structure morphogenesis	6	11	0.00340956
GO:0080003	thalianol metabolic process	3	3	0.00428734
GO:0009411	response to UV	12	36	0.00456944
GO:0010073	meristem maintenance	19	71	0.00457302
GO:0009873	ethylene mediated signaling pathway	26	110	0.00470291
GO:0007000	nucleolus organization	9	23	0.00484539
GO:0002679	respiratory burst involved in defense response	28	122	0.00499171
GO:0016444	somatic cell DNA recombination	11	32	0.00540937
GO:0016568	chromatin modification	10	28	0.0062505
GO:0051753	mannan synthase activity	4	6	0.00799621

Table 3.3 GO analysis of ann2 up-regulated genes

GO term	Description	Overlap Count	GO count	Adj. P-value
GO:0010345	suberin biosynthetic process	7	10	4.30E-05
GO:0016556	mRNA modification	25	100	0.00011399
GO:0008289	lipid binding	24	103	0.00060135
GO:0042335	cuticle development	14	47	0.00102357
GO:0010143	cutin biosynthetic process	6	11	0.00128279
GO:0090447	glycerol-3-phosphate 2-O-acyltransferase activity	4	5	0.00146523
GO:0004091	carboxylesterase activity	26	123	0.00182485
GO:0080039	xyloglucan endotransglucosylase activity	4	6	0.00406533
GO:0033946	xyloglucan-specific endo-beta-1,4-glucanase activity	4	6	0.00406533
GO:0006631	fatty acid metabolic process	9	27	0.00511093
GO:0019825	oxygen binding	40	232	0.00549381
GO:0010103	stomatal complex morphogenesis	27	141	0.00787103
GO:0003959	NADPH dehydrogenase activity	4	7	0.00877528

Table 3.4. GO analysis of *ann2* down-regulated genes				
GO term	Description	Overlap Count	GO count	Adj. P-value
GO:0010200	response to chitin	156	421	4.42E-35
GO:0035556	intracellular signal transduction	59	138	1.28E-16
GO:0009611	response to wounding	100	327	1.81E-15
GO:0009693	ethylene biosynthetic process	50	117	4.24E-14
GO:0002679	respiratory burst involved in defense response	51	122	6.73E-14
GO:0009867	jasmonic acid mediated signaling pathway	84	283	3.64E-12
GO:0009738	abscisic acid mediated signaling pathway	69	217	1.45E-11
GO:0009723	response to ethylene stimulus	76	258	8.04E-11
GO:0009753	response to jasmonic acid stimulus	74	263	1.76E-09
GO:0009863	salicylic acid mediated signaling pathway	53	162	1.97E-09
GO:0009695	jasmonic acid biosynthetic process	46	134	5.30E-09
GO:0052542	defense response by callose deposition	23	47	4.49E-08
GO:0042538	hyperosmotic salinity response	49	160	1.23E-07
GO:0009620	response to fungus	41	124	1.68E-07
GO:0006612	protein targeting to membrane	88	365	1.89E-07
GO:0009414	response to water deprivation	83	339	2.34E-07
GO:0010363	regulation of plant-type hypersensitive response	88	368	2.89E-07
GO:0009873	ethylene mediated signaling pathway	36	110	1.72E-06
GO:0043069	negative regulation of programmed cell death	46	166	9.93E-06
GO:0050832	defense response to fungus	75	329	2.68E-05
GO:0009697	salicylic acid biosynthetic process	53	209	3.20E-05
GO:0071456	cellular response to hypoxia	13	25	3.62E-05

Table 3.4 continued

GO:0009862	systemic acquired resistance, salicylic acid mediated signaling pathway	63	266	4.75E-05
GO:0010310	regulation of hydrogen peroxide metabolic process	48	185	4.75E-05
GO:0031348	negative regulation of defense response	60	268	0.00054876
GO:0000165	MAPK cascade	49	207	0.00060271
GO:0009612	response to mechanical stimulus	18	56	0.00244151
GO:0010112	regulation of systemic acquired resistance	6	10	0.00347072
GO:0045088	regulation of innate immune response	15	44	0.0034905
GO:0009595	detection of biotic stimulus	27	102	0.00406741
GO:0000289	nuclear-transcribed mRNA poly(A) tail shortening	3	3	0.00628006
GO:0048479	style development	3	3	0.00628006
GO:0048480	stigma development	3	3	0.00628006
GO:0004601	peroxidase activity	22	80	0.00733208

3.3 Discussion

Plasmodesmatal transport plays important roles in intercellular signaling pathways in plants, which makes it of great importance to study how PD permeability is regulated. The PD in *ann2* phloem pole pericycle cells show either no or abnormally shaped desmotubules compared to those in wild type. Desmotubules in PD are constricted forms of ER strands physically continuous from cortical ER. They consist of central lumens and a circle of helically arranged globular proteins on the outside surface that link to proteins on the plasma membrane through interactions with intermediate proteins. Most hypothesized functions of desmotubules have not yet been proven, but accumulating evidence shows that desmotubules are targets of plant virus MPs and that the integrity of desmotubules plays an important role in hindering the spread of plant viruses in plants (Tilsner et al., 2011). Many MPs are membrane-associated proteins that can be delivered

to desmotubules through endomembrane systems in plants. The characteristic function of certain MPs is to disrupt plant endomembranes including desmotubules using different mechanisms that are species specific, and this disruption is beneficial for the symplastic movement of viruses. For example, MPs from *Cowpea mosaic virus* (CPMV) and *Grapevine fanleaf virus* (GFLV) can form tubules to replace plant desmotubules and facilitate the movement of whole virions (Laporte et al., 2003; Pouwels et al., 2004).

Three PD types are clarified in Ross-Elliott's work (2017). They are simple PD, funnel shaped PD and branched PD. Funnel PDs are reported to be more efficient in unloading in both bulk flow and diffusion scenarios, compared to simple PD due to lower pressure differential and concentration differential. More funnel PDs were observed in phloem pole pericycle cells of *ann1* roots compared to wild type. Since the PD type in *ann1* cannot explain the diffusion restriction of sugar, two other hypotheses that could account for the restriction are: 1) there are fewer PD in the post-phloem zones of *ann1* roots; 2) the defective diffusion of sugar is only due to increased levels of callose on PD. I propose experiments in chapter 4 to look for answers to these questions.

In GO analysis of RNA-seq data, 11 out of 34 GO categories are about biotic stress responses for *ann2*. Knocking out *AnnAt2* downregulates the expression of genes involved in biotic stress responses. Previously, the root fungus *F. oxysporum* was used to test the role of AnnAt2 in mediating the pathogen response of Arabidopsis. Different depths of hyphae penetration in *ann2* and wild type roots were expected. However, there was no consistent data in three independent experiments. If AnnAt2 affects the integrity of desmotubules, then a plant virus should be used instead of a fungus.

Consistent with multifunctional studies of anneixns, the suppression of *AnnAt1* and *AnnAt2* induces transcriptional changes in multiple signaling pathways. My RNA-seq data may provide additional evidence on the existing functional studies in annexins.

In addition, the RNA-seq analysis indicates potentially novel functions of AnnAt1 and AnnAt2. For *ann1*, 10 out of 73 GO categories are related to chromatin modification, which are down-regulated. Whether knocking out *AnnAt1* induces changes in epigenetic level remain unverified.

3.4 Methods and materials

Plant Materials and Growth Conditions

Arabidopsis thaliana Colombia (Col-0) ecotype was used as the wild type in all experiments. *AnnAt1* (At1g35720) and *AnnAt2* (At5g65020) are studied. Annexin T-DNA insertion mutants including *ann1* and *ann2* were used in this study (Wang et al., 2015). All seeds were surface sterilized by 75% (v/v) ethanol for 1 min and 20% bleach (v/v) for 10 min. After washing 5 times with sterile, deionized water, seeds were sowed on agar plates containing no sucrose. Seeds on agar plates were stratified in darkness at 4 °C for 3 d. Stratified plates were placed vertically in a growth chamber (Percival AR-66 L; light intensity of 275 /μmol m^{-2} s^{-1}, humidity of approximately 80%, 20 °C) in continuous light or in darkness. Unless otherwise noted, chemicals were reagent grade from Sigma-Aldridge Co. (St. Louis, MO).

Transmission electron microscopy

The unloading zone of Arabidopsis roots is located in the stele surrounded by endodermal cells, which is consisted of five cell types: protophloem sieve element, metaphloem sieve element, two companion cells and two phloem pole pericycle cells. According to published work (Ross-Elliott et al., 2017), phloem pole pericycle cells are the repository of unloaded proteins. Small molecules such as sugar are also unloaded into phloem pole pericycle cells. Therefore, PD on cell walls of phloem pole pericycle cells in root post-phloem zones of *ann1* and *ann2* grown without sucrose were observed under TEM.

3-d-old seedlings were used in TEM observation. Fixation of seedlings was as described (Ross-Elliott et al., 2017). Longitudinal sections of root post-phloem regions were observed under Tecnai TEM.

Chapter 4

Unsolved questions and future research

In this chapter 1 will discuss major unsolved questions and experiments that could help answer these questions. In chapter 2, I provided evidence that suppressing the expression of AnnAt1 or AnnAt2 resulted in increased ROS and callose levels in root tips of seedlings grown without sucrose, and I proposed that if these increases also occur in PD they would restrict PD permeability. ROS is documented to regulate callose deposition in PD but it is unclear how this occurs. One of the most immediate unsolved questions is, How does AnnAt1 and AnnAt2 regulate callose deposition and PD permeability? An annexin-like protein in cotton fiber was found to play a role in callose synthesis (Andrawis et al., 1993), but the mechanism by which it did so was not revealed. Given that the diffusion of a small molecule like sucrose is restricted through PD in *ann1* and *ann2* roots, it is highly likely that the symplastic transport of transcription factors in roots would also be hindered, which may cause other phenotypic alterations that have not yet been discovered in the mutant roots. So far, the only true PD mutant described in the literature is *cals3,* which has increased callose accumulation on PD, resulting in defective pleiotropic development in Arabidopsis (Vaten et al., 2011). These considerations raise the question, Are annexin mutants also potential true PD mutants? Here, I propose experiments to look for answers to this important question.

Symplastic transport of SHR and miR165/166 in *ann1-2* and *ann2-1*

Since PD diffusion of sugar is defective, it would be reasonable to hypothesize that symplastic transport of macromolecules such as proteins or miRNA is also restricted in the roots of *ann1-2* and *ann2-1*, and that this change could affect plant growth and development. A good

model to study the plasmodesmatal mobility of macromolecules is the transport of transcription factor SHR and miR165/166 (Helariutta et al., 2000; Nakajima et al., 2001; Carlsbecker et al., 2010; Furuta et al., 2012). Transcription factor SHR is first expressed in vascular tissues of Arabidopsis roots and then moves symplastically to the endodermis, where it interacts with another transcription factor SCR to activate small RNA *MIR165/166*. *MIR165/166* moves in the opposite direction, back to the stele to degrade the *PHB* transcripts that determine xylem cell differentiation in a dose-dependent manner.

Both the s*hr* and *scr* mutants and mutants in which *PHB* is specifically expressed in the stele in a miRNA resistant version show ectopically differentiated metaxylem in the place of protoxylem. This same phenotype could be expected in *ann1-2* and *ann2-1*. To test this prediction, xylem differentiation in the root meristem of *ann1-2* and *ann2-1* could be observed by light microscopy. The symplastic movement of SHR to the endodermis can be detected by the fluorescence of GFP in *ann1-2* and *ann2-1* mutants expressing *pSHR::SHR:GFP*, whereas the distribution of MIR165/166 can be detected by *in situ* hybridization with locked nucleic acid (LNA) probes (Carlsbecker et al., 2010).

Do AnnAt1 or AnnAt2 interact with callose synthase or beta-(1, 3)-glucanase enzymes?

Another way AnnAt1 or AnnAt2 could impact callose deposition would be by interacting with and altering the activity of callose synthase (CalS) or beta-1,3 glucanase (BG) enzymes, whose activities could either increase (CalS) or reduce (BG) callose deposits in plasmodesmata (De Storme and Geelen, 2014). Since ROS accumulation was detected in *ann1-2* and *ann2-1*, which may account for high levels of callose deposited on PD, it would be necessary to investigate whether AnnAt1 or AnnAt2 have direct interactions with either CalS or BG.

Three out of the 12 CalS enzymes in Arabidopsis have been found to be PD-associated and

expressed in roots, whereas 6 out of 50 *Arabidopsis* β-(1,3)-glucanases are PD-associated and expressed in roots (Tilsner et al., 2016). Therefore, all 3 callose synthases and 6 β-(1,3)-glucanases were selected to test the possible interactions with AnnAt1 and AnnAt2 by yeast two-hybrid. The genes and primers are listed in Table 4.1. These experiments are in progress now.

Table 4.1. Genes and primers for yeast two-hybrid

Name	id	F primer	R primer
CalS3	AT5G13000	5'GCCGAATTCATGTCTGCTACGAGAGGAGGT 3'	5'ATTCTCGAGTCATTCCTTGTTTCGAGAAGAGC 3'
CalS7	AT1G06490	5'ATCGCAGGCCATGGAGGCC ATGGCGAGTACTAGTAGTGGTGGA3'	5'ATCGCAGGCCATGGAGGCCTCAAGTCCAAAGGTAATAATGGTTAA3'
CalS10	AT2G36850	5'ATCGCAGGCCATGGAGGCCATGGCTAGGGTTTATAGTAATTGG3'	5'ATCGCAGGCCATGGAGGCC TCAGGTCTCAACATTAGCTCTG3'
PdBG1	AT3G13560	5'GCCGAATTCATGCTGCTTCCGAGATGGTT3'	5'ATTGGATCCCTACAAAAGGCGGTCATGC3'
PdBG2	At2g01630	5'GCCGAATTCATGGCTGCCCTTCTTCTCC3'	5'ATTCTCGAGCTACAAGAATACTAAGGCAATGATCAG3'
AtBG_ppap	AT5G42100	5'GCCGAATTCATGGCTTCTTCTTCTCTGCAG3'	5'ATTGGATCCTTACAACCGAAGCTTGATGATG
	At1g66250	5'GCCGAATTCATGGCTTCTCTTCTCCATCTT3'	5'ATTCTCGAGTCACAAGATATTAGCAACGTTCA3'
	At4g31140	5'GCCGAATTCATGTTGTTCAAAGGTGTTTTTGC3'	5'ATTCTCGAGTCACAGAACAATATACAGACAGATGG3'
	At5g58090	5'GCCGAATTCATGGGTTGGGGTTCGG3'	5'ATTCTCGAGTCAAAAAATAGAGACTGCGATG3'

The significance of my work

Because sugars are the primary products of photosynthesis, their regulatory roles in plants have been intensively studied (Lastdrager et al., 2014; Broeckx et al., 2016; Dobrenel et al., 2016; Li and Sheen, 2016). To fully understand sugar signaling, two key parameters that need to be addressed are the subcellular allocation of sugars and their long-distance transport. The feedback

effect of sugar on photosynthesis is a good example of its long-distance regulatory role. Sugar is transported from source organs to sink organs. Elevated sugar levels in leaves and roots repress photosynthesis, while sugar starvation increases photosynthesis. However, experimental evidence on the regulatory mechanisms of sugar on photosynthesis is scant. My research (chapter 2) provides first-time data on a potential regulatory mechanism by which root sugar levels can regulate leaf photosynthesis: i. e., the post-phloem zone of the root, while functioning as a sugar sink, can sense sugar levels delivered from phloem and regulate photosynthesis in source leaves according to the sugar levels sensed.

An obvious unanswered question in this potential mechanism is what is the signal sent from root to shoot. To answer this question, the transcriptome and proteome of phloem sap from annexin mutants and wild-type plants could be compared in seedlings grown without sugar. Mutants that bypass this regulatory pathway could be screened if they exist.

To the decades of research on plant annexins my work has contributed the discovery of a novel function of annexins in plasmodesmatal transport. Knocking out *AnnAt1* and *AnnAt2* results in altered callose deposition on PD. Before my research, there was only one piece of evidence showing that a cotton fiber annexin could function as a potential CalSC component to help regulate callose synthesis (Andrawis et al., 1993). The experiments I have proposed in this chapter may help clarify the exact role of annexins in callose synthesis.

Despite the technical challenges in studying PD, a lack of PD mutants greatly hinders the progress in related areas such as plasmodesmatal transport and non-autonomous signals. Until now, the only PD mutant described in the literature was *cals3* (Vaten et al., 2011). In *CalS3* gain-of-function mutants, elevated levels of callose is deposited on PD, leading to significant reduction of PD aperture, which restricts the symplastic transport of SHR transcription factor and *MIR165*. By detecting the plasmodesmatal transport of SHR and *MIR165* in annexin mutants *ann1-2* and *ann2-*

1, and by studying the role of AnnAt1 and AnnAt2 in callose synthesis, annexin mutants may be used as additional PD mutants to advance an understanding of how the regulation of PD aperture affects plant growth and development.

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